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Study of the immune response and protective effect against neosporosis elicited by mucosal immunization with *Neospora caninum* antigens

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Constam nesta tese os artigos já publicados, que a seguir se discriminam:

Mucosal and systemic T cell response in mice intragastrically infected with *Neospora caninum* tachyzoites

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Protective effect of intranasal immunization with *Neospora caninum* membrane antigens against murine neosporosis established through the gastrointestinal

Pedro Ferreirinha, Joana Dias, Alexandra Correia, Begoña Pérez-Cabezas, Carlos Santos, Luzia Teixeira, Adília Ribeiro, António Rocha, Manuel Vilanova

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“I don't pretend we have all the answers.
But the questions are certainly worth thinking about.”

Arthur C. Clarke

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Abstract

Neospora caninum is an obligate intracellular protozoan that in recent years has been recognized as a major infectious agent responsible for consecutive abortions in cattle leading to high economic losses. Although the gastrointestinal tract is the natural route of infection in horizontally transmitted neosporosis, the host immune response in the intestinal mucosa following parasite infection is largely uncharacterized. Nevertheless, and due to the unique role of the gut mucosa in the establishment of host infection, the stimulation of a local immune response at this mucosa may be a privileged form for the host to counteract or avoid this infection.

In this thesis a murine model of intragastrically established neosporosis was used to further characterize the host intestinal mucosal immune response following *N. caninum* challenge. The obtained results showed that an early Th1-type immune response is elicited in the intestinal mucosa and associated lymphoid tissue of the infected mice thus confirming that immune cells at this mucosal site can actively contribute to host resistance against *N. caninum*. Taking these results into account, an intranasal immunization protocol was developed with the specific aim of eliciting a mucosal immune response as a means to confer protection against neosporosis established through the gastrointestinal tract. The results presented here showed that this immunization strategy successfully induced a long-lasting mucosal and systemic parasite-specific immune response that conferred protection against *N. caninum* infection. Yet despite the intracellular nature of this parasite, no increased interferon-γ production was observed in the immunized mice following infection. In contrast, our results showed that production of intestinal parasite-agglutinating IgA was elicited by immunization suggesting that it could account for the observed protection. Supporting this hypothesis, it is shown here that the developed intranasal immunization protocol can also confer protection against *N. caninum* infection in mice with compromised cell-mediated immunity.

Lastly, the immunogenicity of the used immunization strategy was assessed in cattle, which mounted a humoral and cellular parasite-specific immune response upon the intranasal immunization

Altogether the studies presented here contributed to the characterization of the immune response in the intestinal mucosa of *N. caninum* infected mice and confirmed that a mucosal immunization strategy is be a viable and robust alternative to achieve host protection against this intracellular pathogen.

Resumo

Neospora caninum é um protozoário intracelular obrigatório que nos últimos anos tem sido reconhecido como um importante agente infeccioso responsável por abortos consecutivos em gado bovino que se traduzem em elevadas perdas económicas. Apesar de o tracto gastrointestinal ser a via de infecção natural do parasita na neosporose transmitida de forma horizontal, a resposta imune do hospedeiro na mucosa intestinal após a infecção encontra-se ainda pouco caracterizada. No entanto, e devido ao papel que mucosa intestinal apresenta no estabelecimento da infecção do hospedeiro por *N. caninum*, a indução de uma resposta imunológica nesta mucosa poderá constituir uma forma privilegiada de o hospedeiro evitar a infecção por este parasita.

Nesta tese, usou-se um modelo de neosporose estabelecida pela via intragástrica em murganhos para melhor caracterizar a resposta imunológica intestinal do hospedeiro desencadeada pela infecção com *N. caninum*. Neste estudo, mostrou-se que é induzida uma resposta imunológica precoce do tipo Th1 na mucosa intestinal e tecidos linfóides associados nos murganhos infectados, confirmando assim que as células imunitárias presentes nesta mucosa contribuem activamente para a resistência do hospedeiro face à infecção por *N. caninum*. Tendo em conta estes resultados, foi desenvolvido um protocolo de imunização intra-nasal com o objectivo específico de induzir uma resposta imunológica em mucosas, como um meio para conferir protecção contra a neosporose estabelecida através do trato gastro-intestinal. Os resultados aqui apresentados mostram que esta estratégia de vacinação foi bem-sucedida na indução de uma resposta imunológica específica, duradoura e protectora do hospedeiro, detectável na mucosa intestinal e de modo sistémico. Apesar da natureza intracelular deste parasita, não foi observado um aumento na produção de interferão-γ nos murganhos imunizados após infecção. No entanto, os resultados obtidos mostraram que a IgA intestinal, cuja produção foi induzida pela imunização, é capaz de aglutinar o parasita sugerindo que a resposta humoral mediada por esta imunoglobulina poderá contribuir para a protecção observada. Apoiando essa hipótese, demonstrou-se que a estratégia de imunização utilizada

também confere protecção contra a infecção por *N. caninum* em murganhos que apresentam imunidade celular comprometida.

Por fim, a imunogenicidade do protocolo de imunização utilizado foi confirmada em bovinos, mostrando que o mesmo foi capaz de induzir uma resposta imune humoral e celular específica contra o parasita nestes animais.

No seu conjunto, os resultados aqui apresentados contribuíram para a caracterização da resposta imune na mucosa intestinal de murganhos infectados por *N. caninum* e demonstraram que uma estratégia de imunização em mucosas pode constituir uma alternativa viável e robusta para conferir protecção contra este patogénio intracelular.

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Abbreviations list

BSA	Bovine serum albumin
BMDM	Bone marrow derived macrophages
cDC	Conventional dendritic cells
CpG	Unmethylated cytosine phosphate guanine DNA sequences
DC	Dendritic cell
GALT	Gut associated lymphoid tissue
IEL	Intraepithelial lymphocytes
iIEL	Induced intraepithelial lymphocytes
nIEL	Natural intraepithelial lymphocytes
Ig	Immunoglobulin
i.g.	Intragastric
i.n.	Intranasal
IL	Interleukin
ILF	Intestinal lavage fluids
IFN-γ	Interferon gamma
i.v.	Intravenous
LP	Lamina propria
MALT	Mucosal associated lymphoid tissue
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
NALT	Nasal associated lymphoid tissue
NcMP	*N. caninum* membrane proteins
NcS	*N. caninum* sonicates
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cells
PP	Peyer's patches
qPCR	Quantitative real-time polymerase chain reaction
SD	Standard deviation
SEM	Standard error of the mean
Th	T helper
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alfa
Treg	T regulatory
VLF	Vaginal lavage fluids

Thesis Outline

The present thesis is organized in 8 chapters:

In chapter 1, an overview of the literature relevant for the understanding of the thesis objectives and results is presented. In this regard this chapter contains a description of *N. caninum* life cycle and its implications in the context of infection and pathogenesis, as well as a comprehensive review of the immune related host parasite-relationship and its impact for the development of vaccination strategies against this pathogen. Due to the importance of the intestinal mucosa for the establishment of natural *N. caninum* infection, a summary of the major components of the host immune response in this mucosa as well as their implication for mucosal immunization are also presented.

In chapter 2, the aims of this dissertation are given.

The results derived from the experimental work performed in the context of this dissertation are presented in chapters 3 to 7. Each chapter follows the usual organization of a scientific article, containing an abstract, a brief introduction relevant for the presented results, followed by methods and results sections, finishing with a discussion of the study.

Regarding each results chapter:

Chapter 3 contains part of the results present in the published article entitled: “Mucosal and systemic T cell response in mice intragastrically infected with *Neospora caninum* tachyzoites”, which I co-authored.

Chapter 4 is based in the published article entitled: “Protective effect of intranasal immunization with *Neospora caninum* membrane antigens against murine neosporosis established through the gastrointestinal tract” of which I am the first author. However novel results obtained after its publication that have complemented the published work are also presented in this chapter.

Chapters 5 to 7 contain unpublished results that further explore the protective effect of the intranasal immunization protocol presented in chapter 4 and assess its immunogenicity in cattle.

Lastly, chapter 8 comprises a general discussion of the work presented in this dissertation where the models presented in this thesis are further discussed and future perspectives are proposed.

The articles referred above, that constitute the basis of the results presented in chapters 3 and 4, are annexed in their published form in the end of this dissertation.

Chapter 1

Introduction

1.1 *Neospora caninum*

1.1.1 Phylogeny and identification

Neospora caninum is an obligate intracellular coccidian protozoan belonging to the phylum *apicomplexa* within the *sarcocystidae* family[1,2]. This phylum is a large and diverse group that encompasses over 5000 different species with a wide environmental distribution[3,4]. The name apicomplexa derives from the presence of an evolutionary unique complex structure of organelles located in the cell's apical end, which are essential for host cell invasion by apicomplexa members[5,6]. All the members of this phylum display a characteristic parasitic lifestyle with often complex life cycles that alternate between sexual and asexual reproduction and usually require the infection of more than one host to complete it[4,5]. The host range presented by the members of this phylum is extremely large and varies from aquatic as well as terrestrial invertebrates and vertebrates[5]. Of notice, many of these protozoa are known human pathogens such as *Plasmodium* spp., *Toxoplasma gondii* and *Cryptosporidium* spp.[4,5,7], all of which present a serious concern to public health especially in developing countries. In addition, several members of this phylum are also important pathogens of veterinary relevance like *N. caninum* and also *T. gondii* as well as *Babesia, Theileria* and *Eimeria* spp.[4,5,7] all responsible for high economic losses. Still, it is estimated that only a small fraction of the members of this phylum has been discovered[3,4] and therefore the number of hosts affected by *apicomplexa* pathogens may be presently underestimated.

Retrospectively, the first described case of neosporosis (the disease caused by *N. caninum* infection) was reported in Norway, in 1984, in dogs suffering from several neuromuscular disorders. In this instance, several parasites with apicomplexa morphology could be observed, however their exact species could not be determined[8]. A few years after, in 1988, *N. caninum* was isolated for the first time in a dog presenting clinical signs of neosporosis[9] and was subsequently described and characterized as a new species in the same year[10]. The relatively recent discovery of this parasite can in part be explained by its

almost identical morphological characteristics to those of *T. gondii*. In fact, it is estimated that before 1988 cases *of N. caninum* infection were largely misidentified and attributed to *T. gondii* since these two parasites are indistinguishable under most microscopic observations[11]. Nevertheless, phylogenetic analysis and comparison of the RNA sequences present in the 18S ribosomal unit of both coccidians proved that these two parasites are indeed separate species that however have only recently diverged[12]. Still, despite all the similarities *N. caninum* presents a unique set of characteristics such as host range, route of transmission and antigenic composition[1] that will be further elaborated in the following chapters.

1.1.2 Cell structure

The life cycle of *N. caninum* is characterized by three distinct infectious-stages: tachyzoite, bradyzoite (organized in tissue cysts) and oocyst, all off each are essential to the parasite survival[13]. Oocysts are ovoid structures with approximately 10 to 15 µm and represent the sexual replication stage of the parasite. Oocysts are initially formed in an unsporulated state that sporulates in 24 hours upon release from the host. The sporulated state is usually characterized by the presence of two sporocysts each containing 4 sporozoites[14,15]. However, very little is known regarding the resistance of oocysts to environmental conditions once outside of the host[16] however, and in analogy with *T. gondii*, they are expected to be very resistant[13,17].

Tachyzoites and bradyzoites replicate asexually and are both intracellular stages of the parasite. Tachyzoites are the most well characterized life-stage of *N. caninum* since these are easier to maintain *in vitro* and isolate from infected hosts. Morphologically, tachyzoites are lunar shaped or ovoid, depending on the state of division, with approximately 6 × 2 µm and represent the acute infection stage of the parasite, capable of fast replication[13,18]. Tachyzoites can infect a wide range of nucleated cells such as neurons, schwann cells, astrocytes, monocytes, fibroblasts, muscle cells, placental cells and hepatocytes[11,19]. This parasitic stage also presents the complex apical vesicle system characteristic of apicomplexa

parasites, composed by micronemes, rhoptries and dense granules. In addition, *N. caninum* tachyzoites also possess a locomotion system powered by an actin-myosin motor named gliding motility that allows parasite mobility and is essential for the process of active invasion of new host cells[11,19].

Bradyzoites are slender shaped with approximately 7 × 2 µm and organized themselves into intracellular tissue cysts[13,18]. Morphologically, bradyzoites closely resemble tachyzoites, presenting however fewer vesicles in the apical complex[11,19]. Tissue cysts can contain several dozen bradyzoites surrounded by a cyst wall and can be primarily found in the central nervous system and muscle cells[1,17]. In this stage the parasite replicates slowly and is capable of maintaining the infection for long periods of time, possibly spawning the entire host's life[13,18]. Nevertheless, and despite the capacity to evade complete sterilization by the host immune response, cysts very rarely lead to clinical manifestations in immunocompetent hosts[2,11].

1.1.3 Host cell invasion

Due to the intracellular lifestyle of apicomplexa parasites, the invasion of new host cells is of paramount importance. In this regard, apicomplexa parasites are capable of actively invade new cells by employing generally conserved mechanisms among this phylum[6,20]. Due to this fact, much of the knowledge on how these parasites invade new cells is derived from studies using *T. gondii* as a model[21]. The first steps of invasion are mediated by a low affinity interaction between the parasite membrane proteins and host cell membrane that allows the initial attachment. Upon this first interaction the parasite reorients itself and sequentially releases the apical vesicles contents, which mediate the process of active invasion. First, microneme proteins are released which stabilizes parasite adhesion. Then rhoptries proteins are ejected, and in conjunction with microneme proteins form a tight ring-like junction that coupled with the gliding motility action of the parasite allows its entry in the host cell. Once inside, the parasite resides in a unique cell compartment designated as the parasitophorus vacuole. At this point the remaining rhoptries and dense granule proteins are released, modulating the

environment of the vacuole and of the host cell, favoring the parasite survival and preventing events such as lysosome fusion[5,6,21].

In the case of *N. caninum*, it has been shown that the parasite can invade a host cell in as little as 5 minutes after the initial attachment[22]. This initial interaction between cells is largely mediated by two major immunodominant proteins in *N. caninum* tachyzoite cell surface, NcSAG1 and NcSRS2, which exhibit high homology with previously identified *T. gondii* proteins also involved in cell adhesion[23]. The importance of these two proteins in parasite survival has been demonstrated *in vitro* where specific antibodies against these proteins were capable of reducing *N. caninum* invasion of new host cells[24-26]. Nevertheless, these initial interactions constitute a very dynamic process and this attachment can be disengaged if the host cell is not suitable for parasite infection or growth[23].

Several proteins from *N. caninum* micronemes have also been identified, once again showing a great degree of homology with *T. gondii* counterparts. As such, *N. caninum* microneme proteins NcMIC1 to 4 have been implicated in the stabilization of the initial interaction between parasite and host cell[27-31]. Similar to the previously mentioned membrane proteins, specific antibodies raised against these microneme proteins were also capable of preventing *N. caninum* host cell invasion[28,29,32]. On the other hand, and in contrast with *T. gondii*, *in vitro* assays have shown that some of these microneme proteins are released spontaneously upon egress from host cell[27-30] which can in part explain the rapid loss of infectivity observed in *N. caninum* tachyzoites after as few as 6 hours of extracellular maintenance[33]. Upon stabilization of the parasite-host cell interaction, rhoptries proteins in conjunction with the microneme protein NcAMA1[34] and the parasite gliding motility[22] mediate *N. caninum* active invasion process[33]. Very few proteins from rhoptries and dense granules have been identified in *N. caninum*. Still, the rhoptry protein NcROP2 is known to be essential for parasite invasion of host cells[35] although its precise role in this process is still unknown[36]. Once inside the parasitophorus vacuole the *N. caninum* tachyzoite and bradyzoite stages divide by endodyogeny[11]. Similarly to *T. gondii, N. caninum* can modulate the host cell environment in order to favor parasite survival. *N. caninum* tachyzoites were recently found to be capable of recruiting mitochondria, the endoplasmic reticulum and late endocytic organelles to the vicinity of the parasitophorus vacuole all of

which the parasite scavenges for nutrients[37]. Further modulation of the host cell environment by the parasite is characterized by the blocking of apoptosis of the host cell favoring *N. caninum* propagation[38].

1.1.4 Host range, life cycle and transmission

Like many members of the phylum apicomplexa, *N. caninum* has a heteroxenous life-cycle and therefore requires both a definitive and an intermediate host to complete it (Figure 1-1). Dogs were the first to be identified as definitive hosts for the parasite[14]. More recently other canine species like coyotes[39], wolfs[40] and dingoes[41] have also been characterized as definitive hosts. On the other hand the parasite can infect a wide range of intermediate hosts, usually large herbivores. Due to the characteristics of *N. caninum* transmission and economic impact, cattle is the most widely recognized and important intermediate host but the parasite has also been isolated from goats, horses and sheep[13,42]. In addition, *N. caninum* has also been isolated in wild herbivores such as white-tail deer, water buffalos and bisons. However, parasite DNA or specific antibodies have been reported in an increasing number of animal species like rats and mice, chicken, rabbit, red fox, rhinoceros, pigs and even dolphins[13,42]. Still, despite its close relation with *T. gondii*, no firm evidence exists that *N. caninum* can infect humans where only low antibody levels have been reported and no DNA or viable parasites have ever been detected[43-45]. Nevertheless experimental infection of Rhesus monkeys has been proven possible and reproducing some of the characteristics of neosporosis[46].

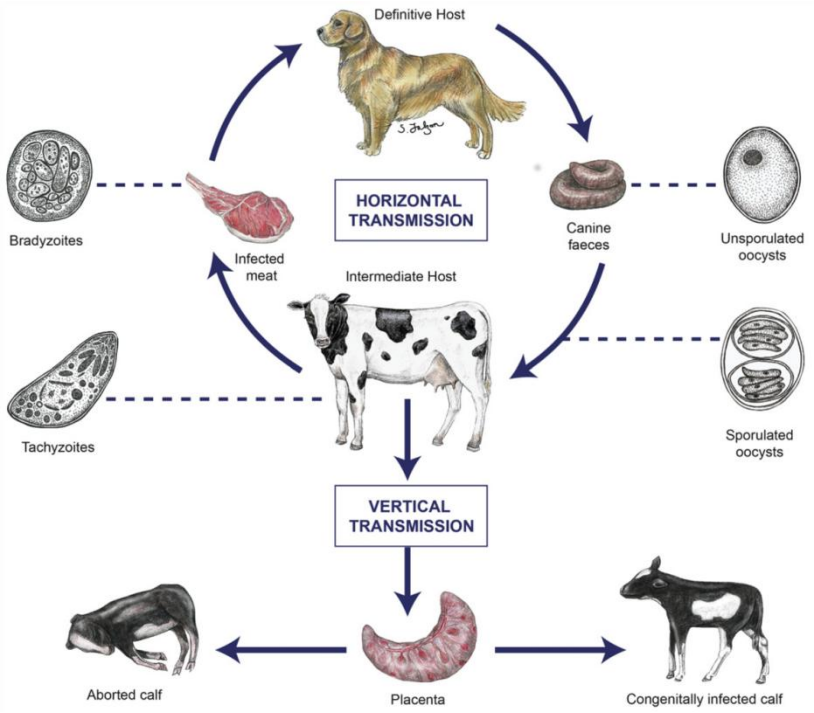

Figure 1-1 – *N. caninum* life cycle. From Goodswen, S *et al*., Infection, Genetics and Evolution, 2013, 13; 133-50

The distinction between definitive and intermediate hosts is tightly related with the cycle of sexual and asexual reproduction of the parasite. As such, in the definitive host the parasite can replicate both sexually and asexually. It is assumed, although it has not been shown, that sexual reproduction occurs in the intestinal epithelial cells of the definitive host[47]. Nevertheless, it is well established that the newly formed oocysts are excreted with the host feces[14,15,48,49]. At least in the case of experimentally infected dogs the shedding of oocysts occurs in a limited time frame[14,15]. However relapsing and *de novo* release of oocysts can occur either by reactivation of a latent infection or by re-infection[49]. Intermediate hosts can become infected through the ingestion of pasture and water contaminated with oocysts. Although not formally shown, but in analogy with *T. gondii*[50], sporozoites are thought to be excystized from the oocysts in the gut of the host, after which they can invade the intestinal epithelial cells and differentiate in tachyzoites[2]. Intracellular tachyzoites rapidly proliferate and are released following induction of host cell lysis[11]. This period marks the acute stage of infection which roughly accounts for the first 2 weeks following initial parasite exposure[2]. Following this period the host is considered latently infected which

coincides with parasite encystation and differentiation into bradyzoites. The parasite life cycle is completed with the infection of a new definitive host through the ingestion of contaminated tissue from an infected intermediate host[51-53]. The transmission between definitive and intermediate host characterizes the horizontal route of transmission of the parasite. Nevertheless, *N. caninum* can also be transmitted vertically (or transplacentally) from dam to fetus during pregnancy. In this case, and depending on the timing when infection occurs, two distinct scenarios can take place: endogenous transplacental transmission or exogenous transplacental transmission[54]. In the first case the primary infection of the progenitor occurs during pregnancy, which leads to parasite dissemination to the fetus during the acute stage of infection. In the second case the parasite is transmitted to the fetus following the recrudescence of a latent infection acquired by the progenitor prior to the pregnancy[54]. Both scenarios have different impacts in the outcome of pregnancy and to the host immune response[13,17,47,54,55].

Transmission of the parasite between definitive and intermediate hosts is best characterized in cattle and dogs due to the relevance of these species as hosts for *N. caninum* and due to the associated pathologies of neosporosis[13,17,55]. Vertical transmission is widely considered the most important transmission route for the maintenance of *N. caninum* infection in cattle herds[56-59]. Several studies have shown that the rate of vertical transmission varies between 61 and 95%[57,60-65] which makes *N. caninum* one of the most successful vertically transmitted pathogens in cattle[13,42]. In addition the parasite can be transmitted over consecutive pregnancies increasing its capacity to perpetuate the infection over several generations[58,66]. Nevertheless, and despite the very high rate of vertical transmission, it is evident from mathematical models that the maintenance of *N. caninum* infection in a herd cannot be sustained without horizontal transmission events[67]. In fact, experimental infection of cattle with oocysts was proven possible[68,69] and vertical transmission could also be observed in some instances[70,71] although endogenous transplacental transmission was not registered in following pregnancies[71]. Further indication of post-natal exposure to the parasite in nature is provided by the existence of seropositive calves born from seronegative dams[72-74] as well as a tendency for increase seropositivity with age[73,75]. In line with these observations, the existence of dogs near cattle livestock

farms has been associated with increased seroprevalence[64,76-78]. In turn, several epidemiological studies have also provided evidence that the distribution profile of infection in herds is consistent with the existence of a point-source exposure to the parasite confirming the existence of horizontal transmission events[58,79-81]. Furthermore, horizontal transmission has been shown to occur regularly in some studies[62,82,83] reaching a mean annual rate of transmission between 22%[62] and 49%[84].

The ingestion of oocysts is so far the only known mode of horizontal transmission in cattle[17,18,55], and as such direct transmission of the parasite by animal contact has not yet been observed[59]. While parasite DNA has been detected in the milk[85] and semen[86,87] of infected cattle direct feeding of calves and dogs with milk spiked tachyzoites was found to be non-infective[51,88] and to date no evidence was found of venereal transmitted neosporosis[89,90].

1.1.5 Neosporosis – pathogenesis

Despite *N. caninum* large range of hosts, neosporosis is a disease observed mainly in dogs and particularly in cattle. The pathologies and clinical manifestations of neosporosis are for the most part a direct consequence of the parasite life-cycle and transmission[17,55]. As previously mentioned, neosporosis was initially described in dogs and the first descriptions of the disease coincide with the discovery of the parasite[8-10]. Clinical signs range from dermatitis, neuromuscular disorders and pneumonia[18,91,92]. However, the most frequent clinical manifestations in dogs are the occurrence of abortions or the birth of congenitally infected animals[93]. In fact the most severe cases of neuromuscular disorders are observed in congenitally infected puppies usually presenting ascending paralysis of the hindlimbs[18,91,92]. Nevertheless, vertical transmission in dogs is highly variable and clinical symptoms in the definitive host seems to be the exception rather than the norm[13,42].

As initially stated, neosporosis is mainly a disease of cattle and most of the pathogenesis in these animals is linked to the parasite transmission. Infected cattle has been reported worldwide although the prevalence of infection can vary

between different countries[13,42] and different cattle breeds[94,95]. Adult, non-pregnant *N. caninum* infected cattle are for the most part asymptomatic, which makes it difficult to identify infected animals[17,42]. However, and as previously mentioned, infected animals display a very high rate of vertical transmission which accounts for much of the pathology associated with bovine neosporosis. During pregnancy, and either as a result of endogenous or exogenous transplacental transmission, the parasite can reach and infect the placenta[96-98]. This process is essential for the parasite crossing of the dam/fetus barrier and establishment of an *in utero* infection. In most instances, *in utero* infection leads to the birth of a congenitally infected and asymptomatic calf[57,60,61]. Nevertheless, a small proportion of congenitally infected animals may develop clinical manifestations in the first months after birth[42,55], usually characterized by several neuromuscular disorders and low body weight gain[99-101].

Although in most cases vertical transmission culminates in the birth of a congenitally infected but otherwise healthy calf, parasite infection of the placenta and fetus can also result in the most common clinical manifestation of bovine neosporosis, which is abortion. In fact, *N. caninum* was recognized as a major pathogen responsible for abortions in cattle very soon after its description[102-106] and isolated from an aborted fetus shortly after[107]. In recent years, the impact of neosporosis associated abortion has been widely recognized since *N. caninum* is regarded as the most common abortive pathogen in cattle[13,42]. Infected dams have a significant increase in abortion risk that is estimated to be between 2 and 7 fold higher in some studies[58,65,72,108,109], while other studies observed a 12 to 13% increase[110,111]. Abortions caused by *N. caninum* arise from irreparable damage to the placenta or fetal tissue[96,112] and may occur year round and in cows of any age[13,55]. Abortions are possible from the third month of gestation until term and although the majority of cases are reported between the 4 and 7 months[78,102-104,106,111,113] the outcome of the fetus infection may vary depending on several factors such as virulence of the *N. caninum* strain and time of infection[42,112,114]. In this regard, infection in early gestation can lead to fetus mummification or resorption while mid-gestation infections often result in the expulsion of an aborted fetus[105,106,115] and late-gestation infections generally end in the birth of a congenitally infected calf[55].

The abortion pattern in *N. caninum* infected herds can be classified as endemic or epidemic. Epidemic outbreaks are by far the most severe events and are often named “abortion storms”[47]. These outbreaks are normally self-contained temporary episodes where at least 15% of the herd dams are at risk of aborting within 4 weeks[47,55]. In severe cases it has been reported that up to 57% of pregnant cows within a herd can abort during an epidemic outbreak[77,80,108,116]. Several studies have indicated that abortion storms are correlated with horizontal transmission events which in part can explain their temporary nature[77,80,81]. This correlation is supported by evidence showing that during epidemic cases dams present low avidity parasite-specific IgG responses indicating a recent exposure to the parasite[62], while the presence of dogs near cattle farms can also be associated with the beginning of some outbreak events[77,81].

After an epidemic event the heard can experience endemic cases of abortion[62,108]. These cases are correlated with endogenous vertical transmission and can occur unpredictably and persist for several months or years[13,42,55]. In fact, during endemic abortion events an increase in parasite-specific antibodies has been reported during pregnancy indicating the recrudescence of a latent infection[60,117] which also accounts for the high degree of association between seropositive dams and abortion cases observed during these events [59,64,74,76,85,112,119].

1.1.6 Neosporosis - economic impact and control

Neosporosis is mainly a disease of pregnant cattle and as such most economic losses are associated with abortion events in cattle livestock farms. Recent projections based in publish data from several countries, estimated that globally 46.5 million cows are at risk of aborting as a result of *N. caninum* infection. In this scenario, the estimated mean worldwide economic losses directly linked to neosporosis in cattle are of 1.3 billion US dollars but can be as high as 2.4 billion[118]. The vast majority of these losses are linked with reproductive failure, which results in the loss of the new offspring and the added costs of veterinary intervention[13,119]. Nevertheless, other sources of economic losses such as a

reduced milk production[120-123] and reduced body weight gain[124] in infected animals have also been reported although with much less expression.

Taking into account these losses, different strategies have been proposed to reduce *N. caninum* economic impact. Some of the proposed measures include: test and cull of infected animals, pharmacological treatment, vaccination or simply perform no intervention and live with the disease[125]. More recently an economic model has been put forward to estimate the feasibility of these strategies in the field[126]. According to this model and contrary to what could be expected, performing no intervention in a herd with very low *N. caninum* seroprevalence (up to 10%) is the soundest option in economic terms. Nevertheless, in the case of an epidemic outbreak or if higher seroprevalence is common the losses incurred by this course of action quickly rise[126]. Testing and culling of infected animals has been proven very effective at decreasing the herd seroprevalence and reducing the cases of abortion[127]. Nonetheless this is a time consuming measure and theoretical models[126] as well as field implementations[127] showed that the investment in this strategy far outweigh the losses associated with neosporosis cases. Furthermore, besides testing and culling, additional steps have to be taken to limit the interaction between dogs or other wild canids with cattle, in order to prevent horizontal transmission and reinfection of the herd, further increasing costs[118,128]. Pharmacological treatment to prevent parasite infection or reactivation during pregnancy has also been explored. In fact, several studies have shown that neosporosis could be averted in experimentally infected mice using different pharmacological compounds[129-133]. In addition, one of these compounds was found to have 90% efficacy in the treatment of experimentally infected cattle resulting in the elimination of the parasite[134]. Nevertheless in all studies drug administration was started before or in the moments after infection of the animals and as such no data is available regarding the treatment efficacy in chronically infected animals which are the most common case in farms. Furthermore and taking into account concerns regarding the production of undesirable residues in milk and meat for human consumption as well as a break in production during treatment of infected animals[119,125], pharmacological intervention even with a 90% efficacy would not constitute an viable economically option for *N. caninum* control[126]. Out of all control measures purposed, vaccination (further discussed

below) stands as the most cost-effective strategy to control bovine neosporosis[126]. Up until recently the only commercial vaccine against bovine neosporosis, Bovilis® Neoguard, was available in a few selected countries. However reports indicated that this vaccine could at most confer 50% protection against *N. caninum* induced abortion[135,136]. Nevertheless, even with a limited efficacy, vaccination was found to be the best option to prevent neosporosis associated losses[126]. In addition, since other successful vaccines against parasites, including some *N. caninum* closely related protozoa, are commercially available[137-141], vaccination against neosporosis should also be feasible. In this regard, and with the recent withdrawal from sales of Bovilis® Neoguard, there is a pressing need for the development of an effective vaccine that can prevent both primary infection and abortion.

1.2 *Neospora caninum* host-parasite relationship

1.2.1 Animal models and experimental infection

Much of the knowledge regarding the host-parasite relationship following *N. caninum* infection has been obtained using murine models. The existence of different inbred mouse strains as well as a vast collection of targeted knockout mice, added to the high costs of performing experimental infection in cattle, made mice a prime species to study *N. caninum* host-parasite interaction in laboratory conditions[119]. In fact, experimental infection in mice was established as a model very shortly after *N. caninum* description[142] and vertical transmission and abortion, the most common characteristics of neosporosis, were shown to be reproducible in infected pregnant mice[119,143-145]. Furthermore, different inbred mouse strains showed clear differences regarding host susceptibility to the parasite that could be linked with the elicited immune response in these strains[146].

N. caninum infection of both definitive and intermediate hosts in the wild is initiated with the ingestion of oocysts or tissues cysts. Nevertheless, nearly all experimental infections in mice and cattle are performed using tachyzoites and

either the intraperitoneal, subcutaneous or intravenous route[143]. This point can in part be explained with the difficulty in obtaining viable oocysts that so far have only been isolated from the feces of infected definitive hosts[68,69,71]. Although some protocols for *N. caninum* cyst differentiation *in vitro* have been described[147,148], differentiation into this parasitic stage is achieved by inducing chemical stress in parasite cell cultures, which can compromise parasite virulence. Furthermore, these protocols are characterized by low cysts yields, which are not compatible with large-scale infection studies. In contrast, and as previously mentioned, tachyzoite maintenance and isolation from *in vitro* cell cultures is a well-established technique commonly used in laboratory conditions that better suit the requirements of experimental infection in mice and infection of mice with tachyzoites using the intragastric route is also feasible[149]. Still, despite the fact that this model could better mimic the natural route of infection observed in the wild, to date very few studies to date have used it to analyze and characterize the host immune response following *N. caninum* challenge[149].

1.2.2 *N. caninum* infection and host immune response

N. caninum is an obligate intracellular pathogen, and similarly to other apicomplexa parasites such as *Plasmodium*[150] and the closely related *T. gondii*[151-154], a cellular mediated immune response characterized by a T helper 1 (Th1) phenotype can be expected as essential for host protection. Indeed interleukin (IL)-12 and interferon gamma (IFN-γ), two hallmark cytokines of Th1-type immune responses have been shown to have a pivotal role in host resistance against *N. caninum* infection. The host protective role of IFN-γ was initially described in *in vitro* assays were this cytokine was shown to prevent *N. caninum* intracellular proliferation[155]. Following this initial study, the importance of the IL-12/IFN-γ axis in the context of neosporosis was dramatically shown in different mice models where depletion of both cytokines with monoclonal antibodies[156,157] or genetic deficiencies in their production[158-160] or its receptors[161] render mice lethally susceptible to *N. caninum* infection. Still, and as expected, host protection against

N. caninum and the maintenance of the IL-12/IFN-γ axis is dependent on the crosstalk and activation of different innate and adaptive immune cells.

1.2.2.1 Innate immunity

Following *N. caninum* infection, innate immune cells are expected to be among the first to encounter and recognize the parasite. Initial recognition of pathogens by innate immune cells is generally mediated by pattern recognition receptors such as those belonging to the toll-like receptor (TLR) family[162-164]. TLRs recognize a limited number of pathogen associated molecular patterns and in the case of *N. caninum* recognition, signaling trough TLRs has been shown to be essential for host survival[159]. Due to their anatomical positions and role in the immune response, dendritic cells (DC) and macrophages are thought to be among the first to come in contact and recognize the parasite. In both cell types TLR2 has been found to be largely responsible for *N. caninum* recognition. Engagement of this TLR lead to cell activation and maturation while its absence resulted in increased parasitic burden following infection[165]. Upon recognition both cell types are known to uptake *N. caninum* either by phagocytosis or active cell invasion by the parasite[166,167].

DC represent a population of professional antigen presenting cells, that upon pathogen uptake are capable of migrating to the draining lymph nodes where they promote and direct T cell differentiation[168,169]. *In vitro* studies using either DC cell lines or primary mouse DC cultures have shown that following *N. caninum* recognition these cells become activated and maturate[166,167]. In agreement with these observations, both conventional and plasmacytoid DC populations were found activated in the spleen of infected mice as early as 12 hours following *N. caninum* challenge[170]. In these studies, DC activation was phenotypically characterized by an increase expression of major histocompatibility complex class (MHC) II, upregulation of co-stimulatory molecules as well as IL-12 production[166,167,170]. In accordance with this phenotype, *in vitro* studies have also shown that *N. caninum* activated DC were capable of inducing T cell differentiation into a host protective IFN-γ producing Th1 phenotype[167]. So far very few studies

have been performed on bovine DC, however it is known that bovine DC are capable of uptaking and processing the parasite leading to cell activation and upregulation of MHC II expression[171]. Still, despite the host protective role of DC, these are also thought to contribute to parasite dissemination following infection. Supporting this idea, *N. caninum* infected DC were shown to have increased mobility and were able to transverse polarized epithelial cell barriers *in vitro*[172]. In addition, mice injected with parasite loaded DC displayed increased parasitic burdens when compared with direct inoculation of tachyzoites[172]. Furthermore, DC can directly sustain parasite survival in the host since *N. caninum* proliferation has been reported in this cell type[166,167].

Macrophages although activated following *N. caninum* recognition display a reduced capacity to up-regulate the expression of co-stimulatory molecules as well as a reduce production of IL-12 in comparison with DC[167]. Contrastingly, murine macrophages infected with *N. caninum* were show to be unable to drive T cell differentiation[167]. However, *N. caninum* stimulated bovine macrophages were found to induce the production of IFN-γ and IL-17 in bovine T cells[173]. Although parasite proliferation has also been observed in this cell population[167], IFN-γ activated macrophages as well as murine[174] and bovine[175] microglial cells, a population of resident macrophage-like cells in the central nervous system, were shown to be capable of controlling intracellular tachyzoites[158]. More recently macrophages were found essential for mice resistance against *N. caninum* infection[176], stressing the importance of this cell population in the control of parasite dissemination.

Due to their capacity to produce IFN-γ and lyse infected cells natural killer (NK) cells[177] can also be expected to contribute to the early immune response against *N. caninum*. In this regard, *in vitro* IL-2 stimulated bovine NK cells were shown to produce IFN-γ following contact with *N. caninum*-infected host cells or directly with *N. caninum* tachyzoites[178]. *In vivo* studies in cattle have also confirm the host protective role of NK cells and showed that these contribute to the early production of IFN-γ following parasite infection[179].

1.2.2.2 Adaptive immunity

As mentioned above, cell-mediated immunity is essential for host protection against *N. caninum* infection. This has been noticed since depletion of $CD4^+$ and $CD8^+$ T cells was shown to render mice lethally susceptible to *N. caninum* challenge[180]. This study also established the predominant role of $CD4^+$ T cells in host protection against neosporosis, since the depletion of this population resulted in a more severe outcome than when $CD8^+$ T cells alone were depleted[180]. Early reports on the role of $CD4^+$ T cells in bovine neosporosis indicate that this cell population was capable of recognizing several *N. caninum* antigens in infected animals[181] with concomitant proliferation and IFN-γ production in both pregnant[182,183] and non-pregnant cattle[181,184,185]. Furthermore, in cattle orally infected with *N. caninum* oocysts a similar activation of $CD4^+$ T cells was also described, indicating that the observed immune response is independent of the route of infection or parasite stage used[68]. Consistent with the predominant protective role of $CD4^+$ T cells in mice, $CD4^+$ T cells were shown to be the major producers of IFN-γ[179] in experimentally infected calves and this cell population was also found to be cytotoxic and capable of lysing *N. caninum* infected bovine cells through a preforin/granzyme pathway[186].

Regarding the role of $CD8^+$ T cells in neosporosis, and despite the indication that this cell population had a smaller contribution to mice resistance against *N. caninum* challenge, MHC I-deficient mice, lacking $CD8^+$ T cells, were shown to be lethally susceptible to *N. caninum* infection[160]. More recently, IFN-γ production rather than the cytotoxic capacity of $CD8^+$ T cells was shown to mediate the host protective effect of these cells in murine acute neosporosis[187]. Although essential for host protection against *N. caninum* infection, both $CD4^+$ and $CD8^+$ T cells can also contribute to host pathology following *N. caninum* challenge since adoptive transfer of *N. caninum*-primed $CD4^+$ and $CD8^+$ T cells increased immunopathology in recipient naïve mice following *N. caninum* infection[188].

Although *N. caninum* is an obligate intracellular pathogen, B cell-deficient mice were found to be lethally susceptible to infection[189]. In fact, this cell population was found to be activated very early after *N. caninum* challenge and

subsequently proliferate[190]. Some of the effector functions of antibodies that can explain the importance of B cells in the context of *N. caninum* infection are their possible capacity to prevent infection of host cells and opsonophagocytosis of the parasite as well as the initiation of antibody-mediated cell cytotoxicity[191]. In fact, several studies have shown that antibodies directed against membrane proteins as well as some proteins of the apical vesicles of *N. caninum* were capable of blocking host cell infection[24-26,28,29,32,192]. Nevertheless, *in vivo* host protection mediated by antibodies against *N. caninum* has never been demonstrated.

1.2.2.3 Pregnancy and immunity

During pregnancy the immune system of the progenitor is faced with a unique challenge - allowing fetal development without rejection of what is essentially a semi-allograft. In order to cope with this situation an extensive modulation of the mother immune response, especially in the maternal-fetal interface, is initiated[193-195]. This modulation most notably leads to an alteration of the Th1/Th2 balance. In this sense, it is well established that during pregnancy cell mediated immunity, that could damage the placenta and fetus, is down regulated in favor of humoral, T regulatory and Th2-type immune responses[193,194]. In this regard, characteristic Th2 cytokines such as IL-4 and IL-5 as well as the anti-inflammatory cytokines IL-10 and transforming growth factor β (TGF-β), are known to be upregulated in the placenta and surrounding tissues[196]. In sharp contrast Th1 cytokines, especially IFN-γ and tumor necrosis factor α (TNF-α) are known to be detrimental to pregnancy and, in the most severe cases, over-production of these cytokines can lead to abortion[197].

Regarding *N. caninum* infection, it is expected that an alteration in the host-parasite relationship during pregnancy, due to a modulation of the dam immune response, can in part explain the high rate of vertical transmission and abortion caused by *N. caninum*[198]. It is still not well established if abortions induced by neosporosis are a consequence of a robust Th1 immune response against the parasite recrudescence or primary infection during pregnancy or the result of parasite proliferation or rather a combination of both[198]. In addition, conflicting

results also exist regarding the dam and fetus protective immune response against *N. caninum* during pregnancy. In naturally infected calves, recrudescence of the parasite has been registered at mid-gestation followed by an increase in IFN-γ production[98]. However, and in contrast with the known damaging effects of IFN-γ during pregnancy, one study pointed the fact that dams that had reduced IFN-γ production following parasite recrudescence were 15.6 times more likely to abort[199]. Still, and in line with the pregnancy-mediated modulation of the host immune response, in a pregnant mouse model of neosporosis a reduction in the levels IL-12 and IFN-γ concomitant with a higher production of IL-4 has been reported[200,201]. Regarding the occurrence of abortions, studies in both cattle and mice showed that the timing of infection during pregnancy could be directly related with the outcome and pathology[198,202,203]. Experimental infection of dams in either early, mid, or late-gestation period was found to be accompanied by an increase in parasite-specific IFN-γ production by peripheral blood mononuclear cells[182,204-210]. Still, despite this similarity, several studies have reported that experimental infection during early-gestation in both cattle and mice led to a very high rate of abortion[96,112,207,209]. The comparison between cytokines produced in the placenta and surrounding tissues after *N. caninum* infection in the early and late gestational periods also showed that although the profile of cytokine production was similar, a higher production of pro-inflammatory cytokines could be observed in the early-gestation challenge[207,211].

Although most cases of *N. caninum* induced abortion in the wild are reported between 4 and 7 moths of gestation, experimental infection of cattle at mid and late-gestation seldom result in abortion, although vertical transmission has been extensively reported[112,182,206,208,210,212]. Fetal immunocompetence in cattle has been described from day 120 of gestation onwards[213] and in accordance in these data, some studies have shown the existence of a parasite-specific immune response with IFN-γ production in fetus of dams infected at mid and late-gestation[182,205,206,208]. This fetal immune response is thought to be a contributing factor for the reduce abortion rate observed at these time-points[198].

1.3 Immunization strategies against *Neospora caninum*

Vaccination is regarded as the best strategy to manage and possibly prevent cattle neosporosis[126]. It is generally assumed that an ideal vaccine against this protozoan should prevent parasite recrudescence and dissemination during pregnancy, thus preventing vertical transmission, and or prevent the host primary infection altogether[33,143]. Taking into consideration the parasite natural route of infection, an ideal immunization protocol should elicit both systemic and mucosal parasite-specific immunity[33]. Since other vaccines for closely related apicomplexa protozoa are commercially available[137,138,141], there is a consensus on the feasibility to develop an effect vaccine against *N. caninum*[119]. Further indication that a successful immunization protocol against neosporosis can be achieved comes from field observations were infected cattle display a progressive reduction in the abortion risk over consecutive pregnancies indicating that some degree of protection can be acquired[63,109,214]. In addition, other studies pointed out that naturally infected cattle, although prone to vertically transmit the parasite, display a reduced risk of abortion during an outbreak event[80] or when experimentally infected during early-gestation[215]. Furthermore, modulation of the host immune response prior to pregnancy was also found to be a determining factor in the prevention of vertical transmission. Neutralization of IL-4 coupled with *N. caninum*-specific priming of the host immune response prior to pregnancy was shown to prevent vertical transmission in mice while similar modulation of IL-4 production during pregnancy was not successful[216].

Taking into account these observations, several strategies have been pursued with the aim of developing an effective immunization protocol against bovine neosporosis[143,217].

1.3.1 Live and attenuated vaccines

The use of live parasites as a means of conferring protection against neosporosis stems directly from the observation that naturally infected animals

display an increased degree of protection regarding fetal loss. In agreement with these observations, infection with a small inoculum of live and virulent tachyzoites was found to protect mice against a lethal challenge with *N. caninum*[218,219] and to prevent vertical transmission in cattle infected at mid-gestation[185]. Still, the use of live and virulent strains in these studies, although effective at proving the concept, would never be desirable for a live immunization strategy due to the virulence status of the used parasites. As such, several natural and laboratory induced attenuated strains of *N. caninum* have been screened, both in mouse and cattle models, for their possible host protective value. In the case of laboratory induced parasite attenuation using gamma radiation, results in murine models are controversial. While one study indicated that previous exposure to these attenuated tachyzoites could confer protection against a lethal challenge[220], another study using mice reported an increased susceptibility to *N. caninum* infection[190]. In the case of naturally attenuated strains, these are in most cases isolated from congenitally infected non-symptomatic calves and have generally been proven safe in cattle since animals infected with these strains are rarely reported to present abortions or vertical transmission events as a consequence of this initial exposure[114,221,222]. Mice[223-225] and cattle[221,222,226,227] immunization with attenuated strains of the parasite prior to pregnancy have proved that this strategy was very successful at preventing both fetal loss and vertical transmission following infection with virulent strains even when this was performed in the early-gestation period. Still, immunization with an attenuated strain in chronically infected cows was only partially successful in reducing abortion[228], indicating that the modulation of host immune response through immunization is most successful when performed prior to parasite exposure.

Although highly effective in mice and cattle, naturally attenuated strains have considerable drawbacks that have prevented their introduction as a viable commercial vaccine against cattle neosporosis. Although proven safe in several studies, the use of live pathogens for immunization purposes raises multiple concerns such as the possibility of cross contamination with other pathogens as well as the reversion to more virulent status which could compromise host protection[119,143,217]. In economic terms, live vaccination also presents a daunting logistic challenge due to their reduced self-life time that severely hampers storage

and distribution[119,221]. Nevertheless, immunization with live attenuated strains has been successfully implemented against other relevant veterinary apicomplexa parasites like the approved vaccines against *T. gondii*[229] and *Babesia bovis*[141]. In this regard, and since no other vaccine against neosporosis is now commercially available, live vaccination has been gaining support from many researchers which propose this strategy as short-term measure to control bovine neosporosis[230].

1.3.2 Whole parasite lysates vaccines

In order to overcome the limitations and concerns regarding live vaccination, many studies evaluated the use whole *N. caninum* lysates as a means of inducing a host protective immune response against neosporosis. Initial studies reported a high degree of protection conferred by this formulation in a pregnant mouse model of infection where vertical transmission was found to be averted in immunized mice[231,232]. These initial results ultimately led to the creation and introduction of the only ever commercial available vaccine against bovine neosporosis, Bovilis® Neoguard, which used whole parasite lysates and havlogen adjuvant. However, the effectiveness of this vaccine has always been controversial and different field studies revealed that this immunization could only confer at best 50% protection against *N. caninum* induced abortion[135], while in some cases immunized dams presented even higher rates of vertical transmission[136]. These generalized doubts about this vaccine effectiveness ultimately led to its recent withdrawal from the market[128,217].

Following the initial successful report of protection against vertical transmission achieved in mice by using *N. caninum* lysates[232], the efficacy of this formulation with different adjuvants was assessed in both mice and cattle. Still, the effectiveness of whole parasite lysates in vaccine formulation remains controversial. While in some instances host protection has been reported[25,233-236], in other cases no significant reduction in parasite burden, or vertical transmission was achieved[226,227,237-239]. Furthermore, immunization with parasite lysates was also found to be capable of exacerbating pathology in immunized mice following infection rendering these highly susceptible to *N. caninum* challenge[190,240,241]. The

increased pathology observed in these studies might be related to the complex antigenic composition of parasite lysates, and also stresses the fact that adjuvant selection can be of paramount importance to achieve host protection. This last point has been clearly shown in mice immunized with parasite lysates without any adjuvant which greatly exacerbated mice susceptibility to *N. caninum* infection[218] indicating that this antigenic formulation can also induce a host non-protective immune response that cloud account for the lack of protection observed in some studies[33].

1.3.3 Parasite subunit vaccines

The majority of studies published to date aiming at developing an effective immunization protocol against neosporosis have focused on the use of single parasite recombinant proteins[33,119,143,217]. The use of selected proteins or antigens for immunization offers several advantages over the previous discussed alternatives, since these formulations are in most cases considered safe and have a high stability and shelf-life[33,119]. Most proteins used in these studies, such as *N. caninum* surface proteins NcSAG1 and NcSRS2, were selected for their immunodominant status or their importance in key steps in the parasite life cycle, such as adhesion and invasion of host cells[33,217]. Different studies indicate that immunization with recombinant NcSAG1[25,242] or recombinant NcSRS2[25,243-246] conferred protection to mice upon *N. caninum* challenge. Still, in most instances, the achieved protection was characterized by a reduction in parasitic burdens and not clearance of the parasite. This characteristic might account for the relatively modest results obtained in pregnant mouse models where upon infection only small reductions in vertical transmission and fetal loss were observed[25,243]. Nevertheless, and despite the pivotal role of these proteins in *N. caninum* survival[23], the protective effect conferred by these two cell surface proteins is still disputable since a few studies found no increased protection in infected immunized mice despite the induction of an antigen-specific immune response[242,247]. These results once again indicate that adjuvant selection can be a

determining factor to achieve protection even when targeting essential proteins for parasite survival.

Besides NcSAG1 and NcSRS2, several apical vesicle proteins have also been screened for their possible host protective effect. Although microneme proteins NcMIC1, NcMIC3 and NcMIC4 were shown to be involved in parasite adhesion to host cells[28-30], the protection conferred by immunization with these recombinant proteins is once again controversial. While NcMIC3 immunized mice were found to have a small parasite burden reduction[248], a recent study did not observed any significant protection when using this recombinant protein[242]. However, clear differences between the two studies exist regarding the protocol for recombinant protein production that can account for the different outcomes. In the case of NcMIC1 immunized mice, these were reported to have a reduction in parasitic burdens following infection[249], however and in sharp contrast, immunization with recombinant NcMIC4 was largely unsuccessful and even precipitated lethal pathology in immunized mice[32]. Immunization with the microneme protein NcAMA1 also presented promising results in mice since it was successful, to some extent, at preventing parasite colonization as well as fetal loss[250]. On the other hand, several reports in murine models demonstrated the host protective effect rhoptry protein NcROP2 combined with different adjuvants and with other microneme proteins, although once again the observed protection was only characterized by parasitic burden reduction[35,251-253]. The dense granule protein NcGRA7[254] was also shown to confer some level of protection against *N. caninum* in both mice and cattle[255,256]. However, NcGRA7 altogether with the exclusively bradyzoite stage antigen NcSAG4 were found to be non-protective in pregnant and only slightly protective in non-pregnant mouse models[257]. In addition, cattle immunization with recombinant NcGRA7 and NcSAG1 conferred no protection against vertical transmission when experimental infection was performed at early gestation[258]. Other studies have also screen bradyzoite exclusive antigens in the formulation of immunization protocols, but only partial protection was afforded by most of the used proteins[259], while in one study a mixture of bradyzoite and tachyzoite proteins was again found to be completely non-protective[260].

In most studies a mixed Th1/Th2 immune response was elicited following immunization with tachyzoite surface or apical vesicles proteins[35,242,245,250,251,257]. Nevertheless, and despite the ample evidence that a Th1 type immune response is essential for host survival, a Th2 biased immune response was also found to be host protective, in some instances leading to parasitic burden reduction [243,252,253,261,262]. In one particular case, immunization with a NcROP2 and NcMIC1 and 3 chimeric antigen was capable of achieving host protection with the stimulation of a Th2-type immune response[253], while the same antigen used with a Th1 stimulating adjuvant, although successful at inducing a Th1-biased parasite-specific immune response, was found to be non-protective[263]. Interestingly, and despite in this case a host protective Th2 immune response was reported, this chimeric protein was unable to confer protection against vertical transmission and fetal loss in pregnant mice model[264].

Besides the selection of suitable antigens and adjuvant when developing an effective immunization strategy, a recent set of reports also showed that the route of immunization used could be a determining factor in vaccine effectiveness against *N. caninum*. In this sense NcPDI, a tachyzoite cell surface disulfide isomerase protein[192], was found to have host protective capabilities only when administered intranasally in contrast with the induction of a non-protective host immune response when administered intraperitoneally even with different adjuvants[252,261]. Nevertheless, the observed protection in intranasal (i.n.) immunization was only translated into a reduction of parasitic burdens, and although once again a host protective Th2 immune response was elicited in non-pregnant mice, i.n. immunization with NcPDI was found to be non-protective in a pregnant mice model where it elicited a Th1-type immune response[262]. Nonetheless, these studies were the first to employ mucosal immunization as a means to confer protection against *N. caninum*. Despite the fact that natural infection with *N. caninum* occurs through the gastrointestinal mucosa and thus an ideal immunization protocol against this pathogen should induce both mucosal and systemic immunity, the stimulation of a parasite-specific mucosal immune response as a means to confer protection against *N. caninum* has never been attempted.

1.4 Intestinal mucosal immunity

Mucosa associated lymphoid tissue

The host mucosal surfaces collectively account for the majority of the surface area in contact with the outside environment. In fact, the intestinal mucosa alone presents a surface area greater than that of the skin and as such it is no surprise that besides harboring a vast collection of symbiotic commensal microbes that constitute the gastrointestinal microbiota, this mucosa is one of the most common entry sites for several pathogens[265-267]. In this scenario, the intestinal mucosa poses a significant challenge for the host immune system since it needs to maintain a mucosal homeostasis allowing the presence of a large collection of commensal residents while simultaneously respond to and prevent pathogen invasion[267,268]. As such, the immune system maintains at the intestinal mucosa a considerable amount of the host total lymphocyte population organized in lymphoid structures collectively known as the gut associated lymphoid tissue (GALT) that can be spatially separated into two main locations - inductive and effector sites, differentiated by their anatomical and functional proprieties[267-270]

1.4.1 GALT inductive and effector sites

Most GALT inductive sites have histological and cellular characteristics similar to those observed in other secondary lymphoid organs[266,269-271]. However, these inductive sites characteristically lack an efferent lymphatic system, meaning that presented antigens are sampled directly from the intestinal luminal space[266,269,270]. For this reason, intestinal draining mesenteric lymph nodes (MLN), although considered a draining site for antigen experienced presenting cells from the intestinal mucosa, are not considered part of the GALT[269,270]. Antigen sampling from the intestinal lumen, characteristic of GALT inductive sites, is made possible due to the existence of a specialized follicle-associated epithelium over lining these structures. This epithelium contains several adaptations that foster antigen

sampling such as the existence of a thinner mucus layer that allows direct contact with luminal contents[269,272,273] as well as the presence of antigen sampling cells known as M cells[269,273]. These cells present a phagocytic-like capacity that allow them to sample large macromolecules or microorganisms through the epithelium allowing antigen uptake by mononuclear phagocytes such as DC and their traffic GALT inductive sites[273].

Distinct lymphoid structures that make up the GALT inductive sites can be identified along the intestinal mucosa such as Peyer's patches (PP), isolated lymphoid follicles and cryptopatches[267,269,271]. PP are the best characterized GALT structures and constitute a primary site for the induction of mucosal immune response[267,270]. PP are especially effective at inducing the activation and differentiation of IgA-producing B cells. As such, these sites constantly present several germinal centers[267] and although their formation is initiated in fetal life, the contact with intestine commensals has been shown to be essential for the development and normal activity of these structures[274].

Both isolated lymphoid follicles and cryptopatches are smaller lymphoid structures that can be found scattered throughout the intestinal mucosa[267,269,275]. While the precise role of these structures is still debatable, isolated lymphoid follicles display a cellular composition similar to that of PP while cryptopatches are enriched in T cells and antigen presenting cells and are almost devoid of B cells[267,269]. Isolated lymphoid follicles formation is exclusively dependent on the colonization of the intestinal mucosa by commensals and germ free mice have been shown to lack these structures[267,274]. Cryptopatches can be found in some of the epithelium crypts formed by the villus organization and are considered precursors of isolated lymphoid follicles following that can arise following gut colonization[267,274].

Upon activation and differentiation in the inductive sites, lymphocytes migrate to the effector sites in the mucosal epithelium and the epithelium lamina propria[269,270]. While the epithelium contains almost exclusively T cells, generally known as intraepithelial lymphocytes (IEL), the lamina propria (LP) is home to very a large number of antibody producing B and plasma cells as well as T cells and mononuclear phagocytes[269]. Although considered effector sites where immune cells can encounter invading pathogens, terminal differentiation of lymphocytes,

such as B cell differentiation into plasma cells, occurs in the LP[267,270]. In addition, and as explained in the following chapters, absence of GALT inductive sites in mice does not result in the complete absence of antibody producing plasma cells in the intestinal LP, which also indicates that this effector site can support the initiation of antigen-specific immune responses to some extent[276].

1.4.2 GALT mononuclear phagocytes and antigen trafficking

Macrophages and DC constitute the main populations of mononuclear phagocytic cells in the intestinal mucosa and can be found scattered throughout the mucosa epithelium and adjacent LP as well as in the GALT inductive sites[277]. While replenishment of both cell types in the intestinal mucosa is assured by common bone marrow progenitors[277-279], mucosal DC and macrophages were shown to arise from different precursors[280] and fulfill different roles in the tissue. Much due to the expression of several common phenotypic markers, the characterization and distinction of GALT DC and macrophages was until recently highly difficult which sometimes led to misleading characterization of some of their cellular functions[277-279].

Resident intestinal macrophages constitute the majority of mononuclear phagocytes present in GALT effector sites[277]. Even though the exact role fulfilled by these cells is still debatable, possible functions include clearance of invading pathogens, tissue repair and remodeling, and modulation of mucosal immune responses through cytokine production[277,279]. Besides their capacity to uptake antigens trough M cell transport, intestine mucosal macrophages have been shown *in vitro* and *in vivo* to have the capacity to directly sample antigens from the gut lumen[281,282]. However, the implications of antigen uptake by macrophages are still poorly understood since these were shown to be weak inducers of T cell activation and were not found to migrate to GALT inductive sites or MLN following antigen uptake[283]. One possible role attributed to this antigen capture capacity could be the transfer of processed antigens to DC[277].

DC in the intestinal mucosa are mainly responsible for antigen trafficking to inductive sites and initiation of the adaptive immune response[277-279]. DC can also

uptake antigens from M cells in the intestinal epithelium[279], and similar to intestinal macrophages, extend cellular projections through epithelial enterocytes and sample antigens or pathogens directly from the gut lumen[284] (Figure 1-2). Antigen loaded DC preferentially migrate to GALT inductive sites and MLN in a mechanism dependent on the chemokine receptor CCR7[283,285,286]. In the inductive sites, DC were shown to be proficient in lymphocyte activation and responsible for inducing the expression of chemokine receptor CCR9 and the integrin α4β7 expression [285,287], both essential for cellular trafficking and homing of activated lymphocytes to the intestinal mucosa[288,289]. Although GALT DC are known to be capable of supporting the differentiation of Th1[290] and Th17[291] T cell phenotypes *in vitro*, these cells are also key regulators of oral tolerance[286] through the production of TGF-β that stimulate the differentiation and maintenance of T regulatory (Treg) cells[292]. Both the induction of GALT homing markers in activated lymphocytes as well as the biased induction of Treg cell differentiation and oral tolerance to sampled antigens by DC is known to be largely dependent on the production of retinoic acid from dietary vitamin A[288,292]. In this regard, Vitamin A has been shown to induce in DC the expression of the enzymatic machinery necessary for retinoic acid synthesis[287] which in turn can also account for the major role of DC in antigen trafficking and initiation of immune responses in the GALT[279].

1.4.3 GALT effector sites

1.4.3.1 Intestinal intraepithelial lymphocytes

As a GALT effector site, the intestinal epithelium harbors a highly heterogeneous and unique population of resident T cells with different phenotypes and functions generally named IEL[268,293-296]. These lymphocytes are scattered throughout the epithelial barrier at a rate of around 1 IEL per 5 epithelial cells, making them one of the largest immune cell populations in intestinal mucosa[293,296]. The IEL repertoire and functions are highly influenced by the interactions with the nearby intestinal flora and its absence is known to lead to significant alterations in

the IEL population[294]. These cells fulfill several roles in the intestinal epithelium but are mainly involved in tissue remodeling and healing as well as in the rapid control and clearance of invading pathogens[295-297]. In these sense, and in order to allow a swift response IEL usually present several characteristics of both innate and adaptive immune cells, and are normally divided into two main populations[294-296].

Induced IEL (iIEL), are mainly constituted by conventional $CD8^+$ and a small population of $CD4^+$ T cells of thymic origin[294-296] and as such display a repertoire similar to other GALT and circulating T cells[298]. Although these cells can migrate from the thymus directly to GALT effector sites where they become antigen experienced[299], the vast majority of these IEL are activated in GALT inductive sites[294] from where they acquire their intestinal mucosa homing phenotype[288,289]. Once in the epithelium, IEL are known to be permanent resident cells that do not normally recirculate[300,301] and present a characteristic resident memory phenotype [294-296]. As such, these cells are not known to proliferate *in situ* but present however higher cytotoxic and cytokine production capabilities[302]. In this regard, iIEL are known to produce several pro-inflammatory cytokines, including IFN-γ, that are essential for pathogen clearance[294,295]. In fact, this cell population was found to be capable of conferring long-lasting protection against invading pathogens as shown in the case of the apicomplexa parasite *T. gondii*[303].

Natural IEL (nIEL) are comprised of TCRγδ and unconventional $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ T cell populations[294-296]. These unconventional T cells are mainly of thymic origin, acquiring in their differentiation process the intestinal homing molecules and the activation profile characteristic of this population[294,296]. Although also considered resident memory cells, nIEL lack the expression of several activation and memory markers commonly observed in iIEL[294-296]. Even though the majority of these cells are of thymic origin, several reports indicate that low numbers of nIEL can also be found in athymic mice indicating the existence of an extra-thymic differentiation route that can possible occur in cryptopatches[294,296]. nIEL, especially TCRγδ-expressing T cells are highly involved in tissue remodeling and homeostasis[293,294,297]. In this regard, TCRγδ-deficient mice have been shown to present an altered epithelial morphology[304], characterized by deficiencies in epithelial tight junction formation[305]. Still, these $TCR\gamma\delta^+$ T cells are also capable of actively participate in the immune response against invading pathogens[294,297] and

incidentally, TCRγδ$^+$ IEL response has also been shown to synergically contribute to protection against *T. gondii* infection coffered by iIEL[303].

1.4.3.2 Intestinal lamina propria and B cell activation

As previously described, the intestinal LP, which underlines and supports the epithelial barrier constitutes, the second and largest GALT effector site[268-270]. As expected, the LP harbors a multitude of resident immune cells such as several mononuclear phagocytic cells with some intervening T cells and a large population of B cells and immunoglobulin producing plasma cells[269]. In fact, due to the role of antibody producing cells in the maintenance of mucosal homeostasis and protection against invading pathogens, immunoglobulin production and trafficking across the mucosal epithelium is considered a hallmark of mucosal immunity[306,307]. Due to its characteristics and adaptations, IgA is the predominant immunoglobulin isotype present in mucosal secretions and overall the most abundantly produced antibody isotype in the body[308-310]. B cell activation with concomitant class-switch recombination to IgA occurs preferentially in the GALT inductive sites as well as in the MLN[306,309,310] as a consequence of the unique cytokine milieu and cellular composition found in these locations[267]. As expected, the majority of mucosal IgA-producing cells arise from a T cell dependent activation process[306,309] however, T cell independent activation of B cells with IgA class-switching has also been shown to occur in the intestinal mucosa[311].

T cell dependent IgA production occurs mainly in PP and MLN where B cell activation, class-switching and somatic hypermutation takes place in organized lymphoid structures known as germinal centers[309,312] (Figure 1-2). This pathway involves the activation of CD4$^+$ T cells by antigen presenting DC originating from the intestinal mucosa[306,308,309] that favors Treg cell differentiation, as previously remarked, through the production of TGF-β and retinoic acid[292]. Treg cells in PP, and in contrast with other lymphoid organs, have been shown to efficiently differentiate into T follicular helper cells[313] which are essential for T cell dependent B cell activation. The prime role of Treg cells in T cell dependent IgA production can also be explained by their production of TGF-β[314], whose activity and

production has been shown essential for IgA class-switching in B cells[315]. T cell depended IgA production culminates in the synthesis of high antigen affinity IgA by B cells[306,309,316] that has been shown to be fundamental for maintenance of intestinal homeostasis. In this regard, lack of somatic hypermutation associated with T dependent B cell activation in mice, resulted in a deregulation of the intestinal microbiota with expansion of several bacterial types that led to an increase bacterial translocation across the mucosa[317].

Mice deficient in T cells[311,318] or lacking GALT inductive sites[291] retain a significant amount of IgA producing cells in the intestinal LP which indicates the existence of a T cell independent IgA production pathway that appears to be dependent on signals provided by the intestinal microbiota[311,318]: Regardless of the site where T cell independent activation of B cells occurs, DC are known to be essential for this process[306,308-310]. In these regard, both PP[319,320] and LP DC[291] have been shown to be capable of directly present antigen to B cells and induced IgA class-switching in a process dependent on TLR signaling, retinoic acid synthesis and TGF-β production[291,318]. Additionally, both DC and intestinal epithelial cells are known to produce the B cell activating factor and the A proliferation-inducing ligand that further stimulates and promotes T cell independent IgA class-switching[318,321]. As expected, IgA produced from T cell independent activation, also known as natural IgA, presents a low affinity to target antigens[306,316] but nevertheless can efficiently recognize and coat intestinal microbiota residents[322], indicating a role in the modulation of the intestinal flora and in gut homeostasis[310,323].

In both T cell dependent or independent B cell activation, DC, through retinoic acid production, are known to be responsible for the induction of intestine homing markers in newly differentiated IgA producing cells[320]. Once in the LP, B cells terminally differentiate into IgA producing plasma cells[306,309] (Figure 1-2). Although this process is not fully understood, it is believed that this differentiation is dependent on signals provided by both DC and macrophages as well as signals derived from intestinal epithelial cells[306,307]. Once terminally differentiated, IgA producing plasma cells in the intestine LP were shown to be long lived[324], but nevertheless their maintenance is highly dependent on survival signals provided by Treg cells[314]. Even though IgA plasma cells are long-lived, memory IgA

responses at mucosal sites present a relatively rapid turnover. In this regard, a study has shown that upon arrival, new IgA producing cells displace resident plasma cells in the LP in a competition process[324]. Although this process can lead to the relatively rapid loss of IgA producing cells against certain antigens, it is believed that this strategy allows for a dynamic rearrangement of the IgA repertoire that can better adapt to changes in the intestinal flora allowing a stronger response against the most prevalent mucosal antigens[324]. Nevertheless, and particularly in the context of immunization, IgA^+ memory cells are thought to be maintained in the GALT in spite of the reduction of IgA production in the LP overtime[325]. In this regard, and although the number of IgA producing cells decreased overtime in the LP following oral immunization with cholera toxin, mice still presented memory IgA producing antigen-specific cells in MLN one year after infection[326] and following toxin re-exposure a rapid expansion and detection of antigen-specific IgA in the LP could be observed[327], indicating that long-term mucosal immune memory can be achieved.

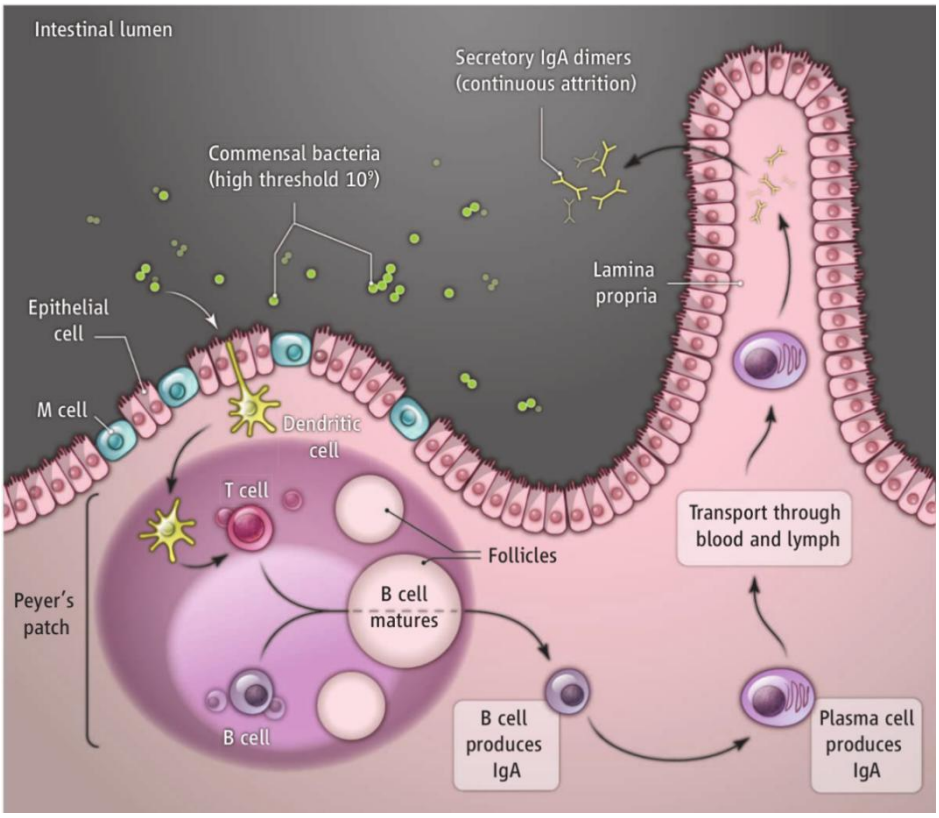

Figure 1-2 – Schematic representation of antigen sampling by GALT DC and lymphocyte activation in GALT inductive sites. Following activation terminal differentiation and IgA

production by B cells occurs in the intestinal lamina propria. From Cerutti, A, Science, 2010, 328; 1646-7

1.4.3.3 IgA secretion and effector functions

Trafficking and secretion of immunoglobulin across the mucosal epithelium is a multistep process involving plasma cells and epithelial cells[266,307-309]. Although IgA is the most abundant antibody produced and secreted at mucosal sites[308-310], IgM can also by trafficked across mucosal barriers although much less efficiently[266]. Upon production, monomeric IgA is dimerized in the LP through the interaction with a small polypeptide produced by plasma cells named joining chain. Upon dimerization, joining chain-containing antibodies can bind to the poly-Ig receptor present in the basal membrane of epithelial cells and bound antibodies are then trafficked across the epithelial cell cytoplasm in a process known as transcytosis. The resulting secreted IgA retains a small peptide fragment of the poly-Ig receptor designated secretory component that covalently binds to the IgA polypeptide chain, thereby stabilizing its conformation while at the same time conferring resistance to enzymatic degradation and also allowing IgA retention in the mucus layer[266,307-309].

Once in the mucosal lumen, secreted IgA functions revolve around the maintenance of mucosal homeostasis and prevention of pathogen crossing of the mucosal barrier in combination with other mucosal defenses[267,309,310,323]. One of the most well characterized mechanisms of protection mediated by secreted IgA is the process of immune exclusion. In this mechanism, due to the dimeric conformation of IgA and to the mucus anchorage capacity provided by the secretory component, bound antigens are efficiently agglutinated and entrapped in the mucus layer, preventing their direct contact with epithelial cells[266,307,309,323]. In addition, IgA is essential for the neutralization of several enteric toxins, such as cholera toxin, by their entrapment in the mucus layer and prevention of their activity[266,307,323]. The transcytosis export process also allows IgA to traffic intracellular pathogens and toxin present inside intestinal epithelial cells or in the LP, back to the mucosal lumen[266,307,323]. Furthermore, IgA can also facilitate the

antigen uptake by M cells through a process not yet fully understood that favors antigen sampling and initiation of immune response against enteric antigens[273,309,310,323].

Although the effector mechanisms of IgA mediated protection have been well characterized in past decades the appearance of mice deficient in IgA production or secretion led to some conflicting results regarding the importance of this immunoglobulin in host protection. Nevertheless, IgA secretion has been shown to be important in the regulation of the intestinal microbiota since its absence can be correlated with an expansion of several bacteria[328,329] while transfer of IgA producing cells was capable of restoring mucosal homeostasis[329]. IgA also regulates bacterial translocation across the epithelium and in its absence an increased trafficking of bacteria to the MLN has been reported[319]. In addition, protection against enteric toxins such as cholera toxin has also been shown to be highly dependent on IgA production[330,331]. Furthermore, following previous exposure to pathogens or immunization, the production of antigen-specific IgA has been shown to be capable of preventing pathogen crossing of the epithelial barrier, further indicating the possible host protective role of IgA in immunization protocols[332,333]. Nevertheless, in basal conditions, mice lacking either IgA production or its secretions do not normally present a very severe phenotype[334,335]. This point can in part be explained by the existence of several compensatory mucosal defense mechanisms[308], since IgA-deficient mice present increase IgM production and transcytosis in the intestine, which can account for the attenuation of the mice phenotype[334]. In addition, lack of IgA was shown to have no impact in the clearance of some mucosal invading pathogens[336] even when infection was preceded by pathogen-specific immunization[331]

1.5 Mucosal immunization

In the last century vaccination has proven itself as one of the great successes of preventive medicine becoming an invaluable means of controlling and reducing the incidence of several infectious diseases[337,338]. Among the different immunization types and formulations, most of the approved vaccines used in both human and veterinary medicine are administered through parenteral routes[337,339-341]. In general, these vaccines induce a primarily humoral systemic immune response that has proven nonetheless highly successful at conferring protection especially against blood borne pathogens and toxins[340-347]. Nevertheless and as previously mentioned, host mucosal tissue account for the majority of the surface area in contact with the outside environment and constitute the most common entry site for several pathogens[265-267]. In this regard, induction of pathogen-specific immune response at mucosal sites could prove a determining factor to achieve protection against disease causing mucosal pathogens[325,338-340,342,343,345] or even prevent pathogen crossing of the mucosal barriers[325,340,342,346]. As such and since mucosal sites present a highly developed and dedicated mucosal associated lymphoid tissue (MALT)[267-270], mucosal immunization can constitute a valuable strategy to induce pathogen-specific mucosal immune responses[325,342,346]. In comparison with parenteral administered vaccines, mucosal immunization strategies present several advantages such as the capacity to induce both mucosal and systemic humoral and cellular immune responses[325,344,346,348], easy and painless administration leading to better vaccination acceptance by the public, reduced risk of cross contamination, absence of invasive procedures and site reactions, reduced costs of purification and no need for highly trained medical and veterinary staff, as well as easier distribution and administration to large scale populations[325,337,339,340,342,343,346,349]. Still, despite the advantages of mucosal vaccination, this strategy has seen limited implementation in both human and veterinary medicine, although the knowledge achieved in recent decades in the field of mucosal immunity has increase the interest in this strategy[340,344,349].

1.5.1 Intestinal mucosa and mucosal immunization sites

Depending on the target mucosa and due to the unique characteristics of the different mucosal sites, the designing of a successful mucosal immunization protocol heavily relies in the selection of the most advantageous mucosal route[339,341]. Due to the large number of pathogens that invade the host through the intestinal mucosa[265-267], the induction of pathogen-specific immune responses at this site has been one of the main goals of mucosal immunization[337,341]. As such, oral administration has been one of the best studied routes for mucosal immunization and has proven that stimulation of the GALT and induction of both mucosal and systemic immune response against target antigens is possible[325]. Nevertheless, and despite in general a stronger mucosal response is elicited in the MALT site of vaccine exposure[325,339], oral immunization has also faced several obstacles that contributed to its limited employment[325,337,346]. Due to the natural physical and chemical barriers of the gastrointestinal tract, oral delivered antigens are subject to the same unspecific host protective mechanism that target invading microorganisms. As such, oral delivered antigens and adjuvants usually display low bioavailability and are subject to high levels of degradation as the result of the high pH variations, high proteolytic activity and entrapment in the mucus layer[325,337,346]. This point is especially important in ruminants where the more complex gastrointestinal system and increased digestion times makes it harder for oral administered vaccine formulations to reach GALT inductive sites[350].

The identification and organization of the MALT in inductive and effector sites where activated cells could recirculate and populate different mucosal effector sites, created the early concept of a common mucosal immune system. Based in on this concept, stimulation of different MALT inductive sites through immunization or direct pathogen encounter could lead to the induction of antigen-specific immune responses at distant mucosal surfaces[344,346,348,349]. In this regard, and due to the limitations presented by oral immunization, the nasal mucosa has also been explored as an alternative for the induction of mucosal immune responses[325,341,346]. The GALT and the nasal associated lymphoid tissue (NALT) share similar characteristics regarding lymphoid tissue organization, specialized epithelium adaptations that allows direct antigen uptake, as well as a similar

immune cell population composition[344,346,348]. Furthermore, and in analogy with the oral route, the nasal mucosa also constitutes an easily accessible site for mucosal immunization that in contrast with the gastrointestinal tract presents increase permeability and absorption rates coupled with limited enzymatic activity, which reduces antigen and adjuvant degradation and increases overall bioavailability[346]. As such, several experimental studies have demonstrated in mouse models that protection against several pathogens could be achieved following i.n. immunization and confirmed the capacity of this route to induce both mucosal and systemic pathogen-specific immune responses[351-359]. i.n. immunization has also been experimentally explored as a vaccination strategy in several veterinary relevant ruminant species[360-367]. In fact, a commercial i.n. vaccine against bovine respiratory syncytial virus in cattle using attenuated virus is already available in the United States (Inforce^{TM}3, Zoetis) and in Europe (Rispoval^{TM}, Zoetis).However, this vaccination employs live attenuated pathogens which raises concerns of pathogen reversion to a more virulent state. Nevertheless, experimental i.n. immunization in ruminants using sub-unit vaccines has shown that this strategy is capable of inducing pathogen-specific mucosal and systemic immune responses characterized by IgA production at mucosal sites, proving the feasibility of using this route in veterinary medicine[362-364,366,367].

More recent data in the study of mucosal immunity suggested the existence of a compartmentalization in different mucosal sites which can compromise the idea of a common mucosal immune system and the possibility of the induction of mucosal immune responses through immunization in distant mucosal sites[339,341,368]. Incidentally, some authors indicate that nasal immunization, although capable of inducing both mucosal and systemic immune responses to target antigens, induces somewhat weak responses in the intestinal mucosa[265,325,344,369]. This fact is linked to the apparent different requirements of cell surface integrins and chemokine receptors for cellular trafficking into the upper respiratory tract and the intestinal mucosa[370]. Still, several experimental i.n. immunization studies have reported conflicting results regarding the description of a stricter compartmentalization of mucosal sites and the apparent dichotomy between the nasal and intestinal mucosa. In this regard, characterization of cell trafficking following i.n. immunization revealed that antigen-specific lymphocytes

can not only be found in NALT effector sites, as well as in the respective draining cervical and mediastinal lymph nodes[371], but also in MLN, spleen and intestinal lamina propria[372]. In addition, activated lymphocytes, and not antigen-loaded DC originating from the nasal mucosa, were shown to migrate to the MLN and intestinal mucosa following i.n. immunization in a process dependent on the expression of the α4β7 integrin[373]. Antigen-specific proliferating T cells in the cervical lymph nodes can upregulate the integrin α4β7[372] and their later dissemination to MLN was demonstrated to be dependent on this expression[373]. The expression of α4β7 has also been implicated in the retention of lymphocytes in the cervical lymph nodes, which can account for the interconnection with intestinal mucosal sites[374]. A recent study has also shown that following i.n. immunization, lung DC were capable of inducing in activated lymphocytes the expression of α4β7 and CCR9 in a process dependent on DC production of TGF-β and the local metabolization of vitamin A into retinoic acid, both of which were essential for lymphocyte migration to the intestinal lamina propria[375]. Furthermore experimental i.n. immunization with SAG1 protein of *T. gondii* revealed that antigen-specific cells could indeed be found in the MLN, and in IEL compartment following immunization and that transfer of these populations to naïve mice could mediate protection against a *T. gondii* challenge[376]. A similar i.n. immunization protocol also indicated that antigen-specific IgA response against *T. gondii* SAG1 could be induced in the intestinal mucosa following i.n. immunization[351,359], even though mice can traffic circulating IgA to the gut lumen through the bile via poly-Ig receptor expression on hepatocytes[266]. Surprisingly, one study has even reported increased protection against helicobacter infection following i.n. immunization in comparison with oral immunization, challenging the idea that i.n. immunization induces weaker responses in the gastrointestinal tract[353]. More recently, reports on ruminants have also shown that following i.n. immunization antigen-specific IgA response could be detected in the animal's feces[363]. A similar gastrointestinal mucosal immune response activation following i.n. immunization was also observed in sheep immunized against serine/threonine phosphatase 2A present in several pathogenic nematode species, which resulted in a reduced presence of viable worms in abomasal contents and reduced shedding of parasitic eggs in the feces[365], indicating that a similar mechanism of cellular trafficking to the

gastrointestinal mucosa following i.n. immunization should also exist in ruminant species.

1.5.2 Mucosal adjuvants

One of the limitations for the large scale introduction of mucosal vaccination is the absence of suitable and licensed mucosal adjuvants[337,342,346,348,377]. In fact aluminum hydroxide salts, the most widely used adjuvant in human vaccination[347], has very little to no adjuvant capacity when used mucosal immunization[342]. In this regard, an ideal mucosal adjuvant should be capable of promoting antigen uptake by mucosal immune cells, present high stability and resistance to pH variations and enzymatic degradation and possess a strong immunostimulatory activity capable of inducing the activation of both B and T lymphocytes, although without causing unwanted immunopathology or inducing tolerance[338,342]. To date the most powerful and well-studied mucosal adjuvants are the bacterial cholera and heat labile toxins[338,342,377]. Although these two bacterial products present very high enteric toxicity, especially in humans, their specific catalytic activity also accounts for their strong mucosal adjuvant capacity[338,342,377]. Both cholera and heat labile toxin are known to be proficient inducers of mucosal IgA responses but nevertheless can stimulate strong cell-mediated immune responses although generally biased towards a Th2 phenotype[325,343,378]. However, and due to its high toxicity, non-toxic derivatives from these two toxins, usually lacking the catalytic domain, have been evaluated for their mucosal adjuvant capacity. These derivatives did not induced significant toxic effects, but presented a considerably reduced adjuvant capacity which limits their use in mucosal immunization protocols[342].

Due to the role of pattern recognition receptors in the activation of innate immune cells, TLR agonists have also been explored as possible adjuvants for mucosal immunization[325,338,342,344,346,347,377]. In this regard, both macrophages and DC as well as other non-related immune cells in mucosal sites express several TLR, making them a valued target for mucosal immune response stimulation[379]. TLR9 is an intracellular pattern recognition receptor that is expressed in multiple

immune cells including conventional and plasmacytoid DC, monocytes, NK cells and B cells and is capable of recognize unmethylated cytosine phosphate guanine DNA sequences (CpG)[347,380,381]. The use of CpG in mucosal immunization has revealed that these sequences possess a strong mucosal immunomodulatory capacity and limited cytotoxic effects[380,382]. Of notice, mucosally administered CpG is capable of inducing strong IgA based mucosal responses as well as Th1-type immune responses which makes it an interesting alternative to conventional toxin-based adjuvants[325,342,346,347,380,381]. Experimentally i.n. immunization with CpG has been shown to induce Th1-type responses against bacterial and protozoan antigens[352-357]. Interestingly, and since CpG administration alone is known to affect the immune response for a considerable time length[381], i.n. immunization with *Yersinia pestis* peptides and CpG adjuvant were capable of inducing a long-lasting production of IgA in the intestinal mucosa indicating that the rapid kinetics of IgA responses can be modulated through mucosal immunization[356]. i.n. immunization with CpG and *T. gondii* sonicates could also induce intestinal IgA production and a systemic Th1-type immune response that were capable of conferring protection against the parasite[357]. Still, despite the advantages and promising results obtained in experimental mouse models, the experimental use of CpG in the development of veterinary related vaccines has been limited. This fact can in part be explained by lack of optimized CpG sequences for some veterinary relevant species. However, and taking into account the promising results obtained in experimental mice models, the use of CpG as a mucosal adjuvant in veterinary medicine may nonetheless contribute for the development of successful mucosal immunization protocols.

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Chapter 2

Objectives

2.1 Objectives

Neospora caninum is one of the leading abortion-inducing pathogens in cattle and therefore responsible for heavy economic losses in cattle livestock farms worldwide. Currently no economically viable strategy is available to control the transmission of this parasite. Although an effective vaccination protocol is regarded as the best cost-effective strategy to prevent neosporosis related abortions, the introduction in the market of an effective vaccine against this protozoan is still not foreseen in the near future.

Although *N. caninum* primary infection is established through the intestinal mucosa, the immune response elicited at this site has largely been uncharacterized. In this regard, the induction of a parasite-specific immune response in the intestinal mucosa, through a mucosal immunization protocol, has never been considered in the literature although it could prove to be a determining factor to achieve host protection against this protozoan. Therefore the specific aims of this thesis were:

1 – To characterize the host immune response elicited in the intestinal mucosa and associated lymphoid tissues of mice infected i.g. with *N. caninum* tachyzoites

2 – To established a mucosal immunization protocol, capable of conferring protection against neosporosis established i.g. in a murine model, with the specific aim of eliciting a parasite-specific mucosal immune response

3 – To assess the immunogenicity of the previously selected mucosal immunization protocol in cattle

Chapter 3

Mucosal and systemic T cell response in mice intragastrically infected with *Neospora caninum* tachyzoites

Mucosal and systemic T cell response in mice intragastrically infected with *Neospora caninum* tachyzoites

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ABSTRACT

The murine model has been widely used to study the host immune response to *Neospora caninum*. However, in most studies, the intraperitoneal route was preferentially used to establish infection. Here, C57BL/6 mice were infected with *N. caninum* tachyzoites by the intragastric route, as it more closely resembles the natural route of infection through the gastrointestinal tract. The elicited T-cell mediated immune response was evaluated in the intestinal epithelium and MLN. Early upon the parasitic challenge, IL-12 production by conventional and plasmacytoid DC was increased in MLN. Accordingly, increased proportions and numbers of TCRαβ^{+}CD8^{+}IFN-γ^{+} lymphocytes were detected, not only in the intestinal epithelium and MLN, but also in the spleen of the infected mice. In this organ, IFN-γ-producing TCRαβ^{+}CD4^{+} T cells were also found to increase in the infected mice, however later than CD8^{+} T cells. These results altogether indicate that following intragastric infection *N. caninum* induces a mucosal and systemic cell-mediated immune response

INTRODUCTION

Neospora caninum is a protozoan parasite found in a wide range of domestic and wild animal hosts[1,2], and is responsible for clinical infections in dogs and cattle[2] having a major impact in dairy and beef industry[3]. Experimentally, the murine model has been the one preferred to study neosporosis, as it presented similar features to the infection occurring naturally in permissive hosts such as brain lesions[4], reproductive loss[5] and mother to fetus parasite transmission[6]. Although *N. caninum* is transplacentally transmitted in cattle with high efficiency[7-12], significant postnatal transmission also occurs in these animals[10,13-15], likely through oocyst ingestion[16]. Even though neosporosis can thus be established through the gastrointestinal tract even in the murine model[17], most studies on the host immune response have been carried out in hosts infected via the intraperitoneal (i.p.) or subcutaneous routes. Consequently, the mucosal immune response to this parasite in infected hosts was barely studied[17]. As mucosal immunizations have been already attempted in experimental models of neosporosis[18-20], the characterization of the immune response to *N. caninum* in the mucosa and associated lymphoid tissues will be helpful to further understand the immunobiology of this parasitic disease. Therefore, a murine model of neosporosis established by intragastric (i.g.) administration of *N. caninum* tachyzoites was used here to study the immune response elicited by this parasite in the gut and associated lymphoid tissue of the infected hosts.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice, 8–10 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific pathogen-free conditions at the Animal Facility of Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Porto, Portugal. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123), 86/609/EEC Directive and Portuguese rules (DL 129/92). Authorization to perform the experiments was issued by the competent national board authority, Direcção Geral de Veterinária (0420/000/000/2008).

Parasites

Neospora caninum tachyzoites (NC-1 isolate) were cultured and serially passaged in VERO cells maintained at 37 °C in Minimum Essential Medium containing Earle's salts (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (200 IU/mL) and streptomycin (200 µg/mL) (all from Sigma) in a humidified atmosphere of 5% CO_2 in air. Free parasitic forms of *N. caninum* were obtained as previously described[17] with slight modifications. Infected VERO cells were cultured until the host cell monolayer was 90% destroyed. Culture supernatants and adherent cells, harvested using a cell scraper, were centrifuged at 1500 × g for 15 min. The pellet was passed through a 25G needle and then washed three times in PBS. The obtained pellet was suspended in 3 mL of PBS and passed through a PD-10 column filled with Sephadex™ G-25 M (Amersham Biosciences Europe GmbH, Freiburg, Germany). Parasite concentration was determined with a haemocytometer.

Challenge infections

N. caninum infections in C57BL/6 mice were performed by the i.g. route using a previously described protocol[17]. Briefly, 5 h before infection mice were deprived of

food. Mice were then anesthetized by intramuscular injection of 20 µL of a 4:5 mixture containing xylazine (Rompum, Bayer Portugal, S.A., Carnaxide) and ketamine (Imalgéne 1000, Bayer Portugal, S.A., Carnaxide). Stomach acidity was neutralized by directly administering into the stomach, with a gavage feeding needle linked to a 1-mL syringe, 50 µL of a 10% sodium bicarbonate solution in water. The same procedure was used to inoculate *N. caninum* tachyzoites 15 min later. Mice were i.g. challenged with 5×10^7 tachyzoites in 0.2 mL of PBS or similarly inoculated with 0.2 mL of PBS and sacrificed at 18 h, 48 h, and 4 and 7 days after challenge.

Sample collection

At the different time points, mice were sacrificed upon isoflurane anesthesia by cervical dislocation and the small intestines, spleens and mesenteric lymph nodes (MLN) were recovered. Spleens and MLN, from infected mice and non-infected controls, were aseptically recovered and homogenized to single cell suspensions in HBSS (Sigma) followed by red blood cell lysis with 0.15 M ammonium chloride for their usage in flow cytometry analysis.

Intraepithelial lymphocyte isolation

Gut intraepithelial lymphocytes (IEL) were isolated as previously described[21]. Briefly, mice small intestines were removed and flushed with 20 mL of cold CMF (Ca^{2+}, Mg^{2+} free HBSS with 1 mM HEPES and 2% FBS, all from Sigma) using a syringe and needle. The Peyer's Patches, fat and remaining mucous were removed along the intestine. The intestine was opened lengthwise, cut into 5 mm pieces and placed in a conical tube with 40 mL CMF. The pieces of tissue were washed twice with CMF by inverting the tube 10 times and letting the pieces settle before removing the supernatant. The intestine pieces were incubated in 25 mL CMF/DTE (CMF with 10% FBS and 1 mM dithioerythritol) (Sigma) at 37 °C and 100 rpm in an orbital incubator (GFL 3031, GFL Burgwedel, Germany) for 20 min. The tube was vortexed at maximum speed for 15 s and the supernatant removed to a new tube. 25 mL of CMF/DTE were added to the tube containing the pieces of tissue and the vortexing step and collection of supernatant were done once more.

All the incubation and supernatant collection steps were repeated. Supernatants from each intestine were pooled and centrifuged at 400 × g, 4 °C for 20 min. The pellet was suspended in 5 mL HBSS with 2% FBS and passed through a nylon wool column pre-wet with HBSS with 2% FBS (0.15 g teased nylon wool in a 5 cc syringe) and the column was washed with 20 mL HBSS with 2% FBS. The collected cell suspension was centrifuged and suspended in 16 mL of 44% Percoll™ (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), adding 8 mL per 14 mL polystyrene round bottom tube. 5 mL of 67% Percoll were underlaid per tube and the 44%/67% Percoll gradients were centrifuged for 20 min at room temperature, 1400 × g, with the brake off. Cells from the interface were carefully removed with a Pasteur pipette and washed twice with 40 mL cold complete RPMI (RPMI 1640 supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin, 1% HEPES buffer, 10% FBS and 0.1 µM β-mercaptoethanol, all from Sigma). Cells were finally suspended in complete RPMI.

Flow cytometric analysis

The assessment of cell surface and cytoplasmic lineage or activation markers on different leukocyte populations was performed by flow cytometric analysis. From spleen, MLN and IEL cell suspensions, prepared as described above, a number of 1×10^6 leucocytes were stained per sample. The following monoclonal antibodies, along with the respective isotype controls were used (at previously determined optimal dilutions) for immunofluorescence cytometric data acquisition in a Coulter EPICS XL flow cytometer (Beckman Coulter, FL, USA): phycoerythrin (PE) anti-mouse TCR β (H57-597) and PE- chlorophyll proteins-cychrome 5 (Cy5) rat anti-mouse CD4 (RM4-5) (both from eBioscience, San Diego, CA, USA); Biotin anti-mouse PDCA-1 (JF05-1C2.4.1) (Miltenyi Biotech, Inc. Auburn, CA, USA); fluorescein isothiocyanate (FITC) hamster anti-mouse CD11c (HL3), PE-Cy5 rat anti-mouse CD8a (53–6.7), Biotin hamster anti-mouse γδ T-cell receptor (GL3), FITC anti-mouse IFN-γ (XMG1.2), (all from BD Pharmingen, San Diego, CA, USA). Biotin conjugated mAbs were revealed with Streptavidin-PE-chlorophyll proteins-cychrome 7 (Cy7) (BD Pharmingen). Cells were pre-incubated for 15 min with anti-FcγR (a kind gift of Dr Jocelyne Demengeot, Gulbenkian Institute of

Science, Oeiras, Portugal). Data were analyzed by using CELLQUEST software (Becton-Dickinson, San Jose, CA, USA).

Intracellular staining

The intracellular expression of the cytokine IFN-γ was detected in splenic and MLN $CD8^+$ and $CD4^+$ T lymphocytes, as well as in IELs (1×10^6 cells), stimulated for 4.5 h with 20 ng/mL phorbol myristate acetate and 200 ng/mL ionomycin in the presence of 10 ng/mL of brefeldin A (all from Sigma). Staining of cell surface markers CD4, CD8, TCR β and TCRγδ was performed as described above, after a pre-incubation step of 15 min with anti-FcγR, followed by fixation in 2% formaldehyde. Cells were permeabilized with 0.5% saponin in flow cytometric buffer (PBS containing 1% BSA and 0.01 M sodium azide) and, subsequently, cells were incubated for 15 min with anti-FcγR and stained for 30 min at room temperature with the appropriate antibody. The intracellular expression of the cytokine IL-12 was assessed in MLN conventional and plasmacytoid dendritic cells (cDC and pDC, respectively). MLN suspensions were enriched in DC by magnetic sorting using anti-CD11c beads (Miltenyi), according to the manufacturer's instructions. 1×10^6 cells were then placed in 96-well tissue culture plates (Nunc) and incubated for 4.5 h with 10 ng/mL of brefeldin A (Sigma). Cells were surface stained with PE-Cy5.5 hamster anti-mouse CD11c (HL3) (BD Pharmingen) and Biotin anti-mouse PDCA-1 (Miltenyi Biotech) revealed with PE-Cy7 conjugated streptavidin (BD Pharmingen). Intracellular staining was performed with PE rat anti-mouse IL-12 (p40/p70) (C15.6) (BD Pharmingen). Intracellular staining with the isotypic controls was performed to confirm the specificity of antibody binding.

Statistical analysis

Unless otherwise indicated, statistical significance of results was determined by unpaired Student t-test, using the GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA). In the scatter dot graphs the mean for each group was displayed as a horizontal line. Column graphs are represented showing mean plus one standard deviation (SD). Results were considered statistically significant with P values of less than 0.05

RESULTS

MLN cDC and pDC produce IL-12 early upon i.g. challenge with *N. caninum* tachyzoites

As we have shown in a previous report, a large proportion of splenic conventional and plasmacytoid dendritic cells (cDC and pDC, respectively) produce IL-12 in BALB/c mice infected i.p. with *N. caninum* tachyzoites[22]. This cytokine is a crucial factor mediating host immune protection against neosporosis[23-25]. As shown in Figure 3-1, an increased frequency of IL-12-expressing cDC and pDC was detected in the MLN of infected mice, 18 and 48 h upon the parasitic challenge. These results show that *N. caninum* tachyzoites administered i.g. induce IL-12 production by host DC in the draining lymph nodes.

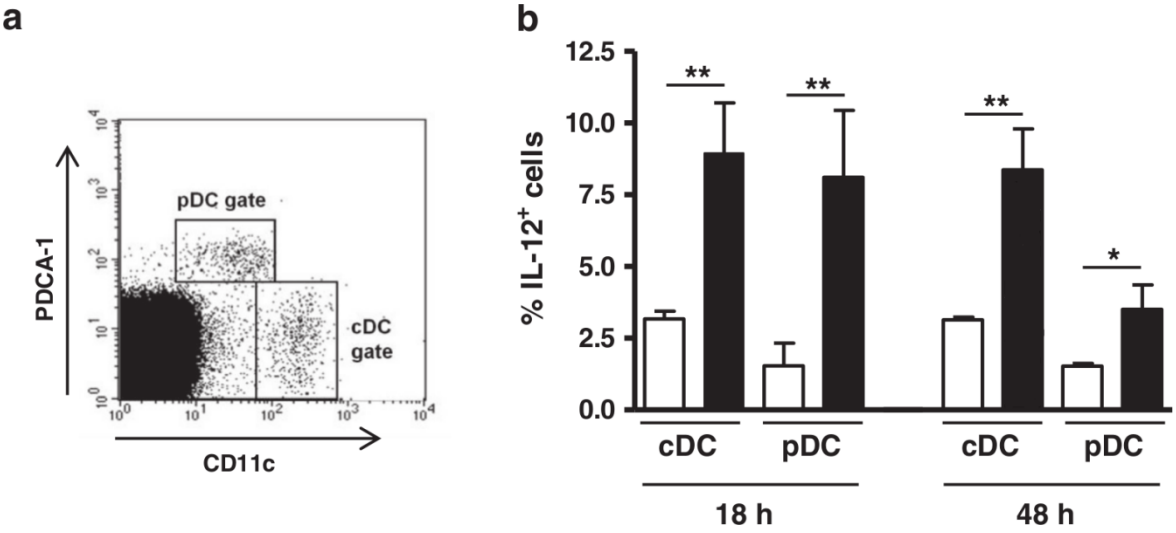

Figure 3-1 - cDC and pDC produce IL-12 in response to *N. caninum* i.g. infection. (a) Representative example of flow cytometric analysis of surface PDCA-1 and CD11c expression on total MLN leukocyte cells. Dot plots represent cells collected from C57BL/6 mice 18 h after i.g. challenge with 5×10^7 *N. caninum* tachyzoites. Gates were set as shown to delineate cDC ($CD11c^{high}$) and pDC ($CD11^{clow}$ PDCA-1^{high}). (b) Proportion of IL-12^+ cells within cDC and pDC populations in the MLN of C57BL/6 mice, evaluated by flow cytometric analysis, at the specified time points after i.g. treatment with PBS (open bars) or i.g. inoculation with 5×10^7 *N. caninum* tachyzoites (closed bars). Bars represent the mean plus one standard deviation of three animals in the PBS group and four animals in the infected mice group. This is one representative result of two independent experiments (*P<0.05; **P<0.01).

Increased frequencies of TCRβ$^+$CD8$^+$IFN-γ$^+$ IEL were observed in C57BL/6 mice challenged i.g. with *N. caninum* tachyzoites

Murine gut IEL comprise both αβ and γδ TCR$^+$ cells[26] which have been shown to mediate host protection against enteric infections, including those caused by protozoans[27]. Here, an increased frequency of TCRβ$^+$CD8$^+$IFN-γ$^+$ IEL was observed in C57BL/6 mice comparatively to mock-infected controls, 48 h upon i.g. challenge with *N. caninum* tachyzoites (Figure 3-2). Conversely, no differences were found between infected mice and controls in the frequencies of IFN-γ-producing TCRγδ$^+$ (1,30 ± 0,27 vs 1,13 ± 0,29) or TCRβ$^+$CD4$^+$ (2,70 ± 0,82 vs 1,84 ± 0,76) IEL. These results indicate that in the gut, TCRαβ$^+$CD8$^+$, but not TCRγδ$^+$, IEL are activated by i.g. administered *N. caninum* tachyzoites and produce the host protective cytokine IFN-γ.

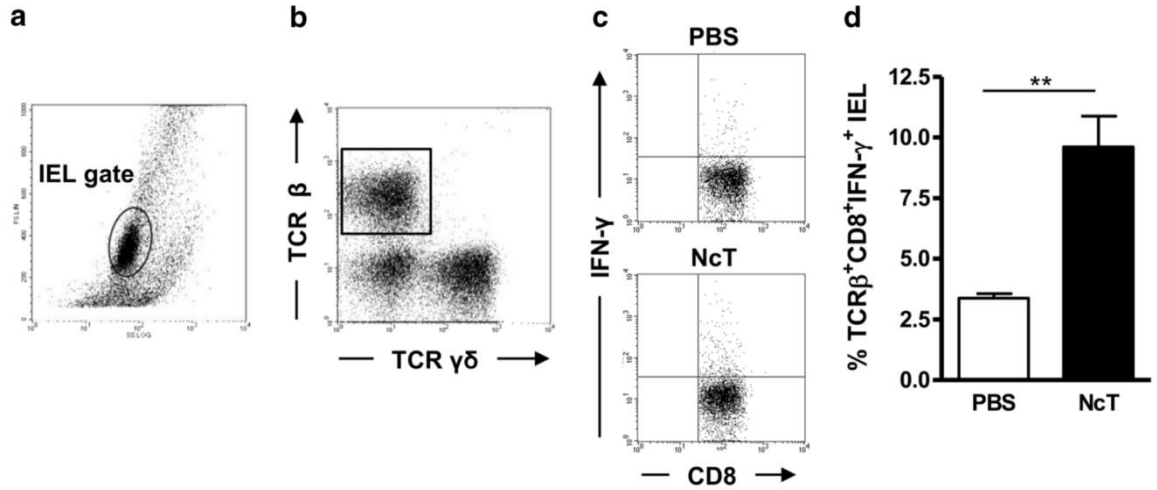

Figure 3-2 - Increased expression of IFN-γ by TCRβ$^+$CD8$^+$ IEL in *N. caninum* infected mice. Representative dot plots of (a) cells isolated from the small intestines of C57BL/6 mice (IEL were gated as shown); (b) TCRγδ and TCRβ IEL. (c) Gated TCRβ$^+$ CD8$^+$ IEL expressing IFN-γ in control (PBS) and *N. caninum*-infected mice (NcT). (d) Frequency of TCRβ$^+$CD8$^+$ IFN-γ$^+$ IEL in control and *N. caninum* i.g.-infected mice, 48 h after challenge. Bars represent mean plus one standard deviation of three animals in the PBS group and four animals in the infected mice group. This is one representative result of three independent experiments (*P<0.05).

CD8$^+$ T cells produce IFN-γ in the MLN of *N. caninum* i.g. infected mice

IL-12 drives the differentiation of T cells towards an IFN-γ-producing phenotype. As increased production of IL-12 was observed in the MLN of *N. caninum* infected mice, the frequency of CD4$^+$ and CD8$^+$ T cells producing IFN-γ were assessed therein, 4 and 7 days upon infection. As shown in Figure 3-3a, an increased frequency of CD8$^+$IFN-γ$^+$ T cells was observed in the MLN of infected mice. This increase was observed at day 4 post-infection whereas at day 7 it was found below control values. No such increase was observed for CD4$^+$IFN-γ$^+$ T cells at the assessed days (Figure 3-3c). Nevertheless, at day 7 upon infection some mice presented CD4$^+$IFN-γ$^+$ T cells at higher frequency than controls. This high variability was observed in the three experiments done. In order to determine whether IFN-γ production could also be induced in splenic T cells upon the i.g. infection, their frequency and number was similarly assessed. As shown in Figure 3-3b, increased proportions of CD8$^+$IFN-γ$^+$ T cells were also observed in the spleen 4 days upon infection that were found within control values by day 7. In contrast, the frequency of CD4$^+$IFN-γ$^+$ T cells, which was similar to controls 4 days after infection, significantly increased at day 7 (Figure 3-3d). These results show that in the i.g. infected mice CD8$^+$ T cells are early producers of IFN-γ, not only at the intestinal mucosa and draining lymph nodes but also systemically, as detected in the spleen.

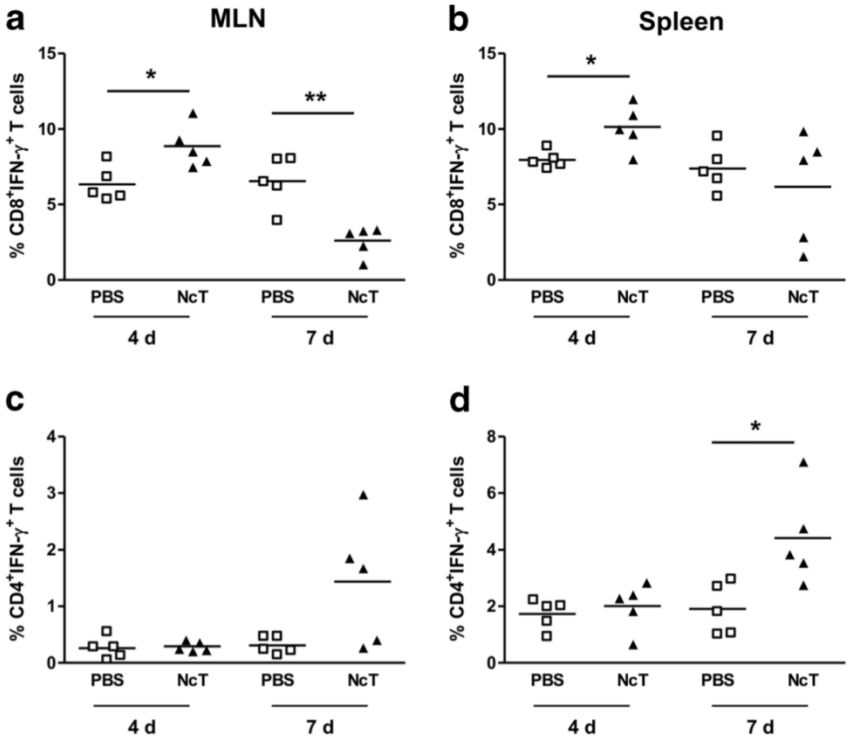

Figure 3-3 - Expression of IFN-γ in splenic and MLN $CD8^+$ and $CD4^+$ T cells. Scatter plots of frequency of $CD8^+$IFN-γ^+ T cells (a and b) or $CD4^+$IFN-γ^+ T cells (c and d), as indicated, in the MLN (a and c) and spleen (b and d) of control (PBS) or *N. caninum*-infected mice (NcT), 4 and 7 days upon i.g. challenge. Each symbol represents an individual mouse; horizontal bars correspond to mean values of the respective group. Five mice per group were used. This is one representative result of three independent experiments. Statistical significance between groups is indicated above symbols (*P<0.05; **P<0.01).

DISCUSSION

Although the gastrointestinal mucosa constitutes the natural route of infection for *N. caninum* in horizontal transmitted neosporosis[2], the study and characterization of the immune response elicited in this mucosa following *N. caninum* infection has been largely overlooked. This fact can in part be explained since experimental infection with the parasite in both cattle and mouse models is usually performed trough parental routes although, at least in the murine model, i.g. infection with *N. caninum* tachyzoites was shown to be feasible[17]. Although this parasitic form may present antigenic differences from oocysts and sporozoites, this model may nevertheless better mimic the natural infection route used by the parasite. In this work we have thus use the i.g. infection model, previously established by our group, to characterize the host mucosal and systemic immune response in the early time-points after mice infection with *N. caninum*. In this regard, analysis of DC in the MLN either 18 or 48h after infection revealed an increased frequency of both cDC and pDC populations producing IL-12, an essential cytokine for host resistance against *N. caninum* infection[23-25]. This result is in agreement with our group's previous observation that both cDC and pDC populations present increase IL-12 production in the spleen of *N. caninum* i.p. infected mice[22] as well as with *in vitro* studies where increase IL-12 production was reported following DC activation with *N. caninum* tachyzoites[28,29]. In addition, similar IL-12 production profiles by cDC and pDC have also been described in mice infected with the closely related apicomplexa parasite *Toxoplasma gondii*[30,31]. On the other hand, and since DC have been implicated in the dissemination through the host of *N. caninum*[32] as well as *T. gondii*[33,34], it would be worth assessing if the analyzed DC found in MLN were locally activated by the parasite or rather migrated from the intestinal mucosa and can thus contribute to parasite dissemination through the host.

Consistent with the early IL-12 production observed in MLN DC, 18 and 48h after infection, higher frequencies of TCRαβ^{+}CD8^{+}IFN-γ^{+} IELs were observed in the intestinal epithelium of infected mice. Due to the paramount role of IFN-γ in host resistance against neosporosis[23,35,36], this early production of IFN-γ by IELs could contribute to the host control of the parasite infection and limit its

dissemination in the intestinal mucosa. In this regard, *T. gondii* primed IELs have been shown to mediate protective immunity in adoptive cell transfer experiments to *T. gondii* oral infected mice[37,38]. In these studies, *T. gondii* primed TCRαβ$^+$CD8$^+$ IEL also presented increase IFN-γ expression and protection conferred by transferred IELs was also shown to be largely mediated by this cell population [37,38]. Still, despite the increased frequency of IELs producing IFN-γ observed in our study, the contribution of these cells for host protection is certainly different in the case of *N. caninum* infection since similar transfer of *N. caninum* primed IELs followed by i.g. challenge of recipient mice with *N. caninum* tachyzoites did not result in increased mice protection when compared with control mice receiving unprimed cells (our group's unpublished results). Although in agreement with the increase IL-12 production by DC in MLN, and even though MLN have been shown to be a major site for IEL activation and differentiation[39], due to the fact that increase IFN-γ in TCRαβ$^+$CD8$^+$ IEL was detected just 48h after infection, it would also be worth assessing if these IFN-γ producing IELs are newly activated and recent emigrants to the intestinal mucosa or resident cells that can be activated quickly *in situ* following parasite encounter.

Also consistent with the early IL-12 production by DC in the MLN and the higher frequency of TCRαβ$^+$CD8$^+$IFN-γ$^+$ IELs, 4 days after infection higher frequencies of CD8$^+$ T cells producing IFN-γ were observed in the MLN and in the spleen of infected mice. This result implicates CD8$^+$ T cells also as early responders to *N. caninum* in both lymphoid organs. In addition, this result also indicates that 4 days after infection a mucosal associated and systemic immune response could be observed in *N. caninum* i.g. infected mice, although we could not discard the possibility that higher frequencies of CD8$^+$ T cells in the spleen could be the result of cell trafficking to this site of cells from other lymphoid organs rather that *in situ* activation. In this sense, analysis of the activation profile of splenic DC could help determine if an active immune response is being undertaken at this site and also determine if infected DC could disseminate the parasite from the intestinal mucosa to the spleen. Nevertheless, and similar to the results observed here, CD8$^+$ T cells were also shown to be early producers of IFN-γ following *T. gondii* infection, and were also involved in host resistance during the acute infection stage[40]. Recently, our group has also shown that CD8$^+$ T cell IFN-γ

production mediates the host protective effect of this cell population in *N. caninum* infected mice[41]. In contrast, similar analysis performed 7 days after infection revealed reduced frequency of $CD8^+$ T cells producing IFN-γ in the MLN and higher frequencies of $CD4^+$IFN-γ^+ T cells in the spleen of infected mice. In this sense, and although $CD4^+$ T cells have been established as the main mediators of host protection following *N. caninum* infection in comparison with $CD8^+$ T cells[42], these appear to be late responders in i.g. established neosporosis. Also worth assessing is the possibility that the apparent reduction in the frequency of IFN-γ producing $CD8^+$ T cells in MLN could be the result of cell migration to effector sites.

Altogether these results show that in i.g. infected mice, *N. caninum* stimulates an early mucosal and systemic immune response with the production of host protective IL-12 and IFN-γ. Furthermore, $CD8^+$ T cells in the intestinal mucosa as well as in the spleen and MLN were shown to be the early responders to *N. caninum* infection. Since the intestinal mucosa is the first site to come into contact with the parasite during horizontal transmitted neosporosis, the knowledge that following i.g. infection *N. caninum* stimulates both a mucosa and systemic immune response can also aid in the development of host protective immunization strategies against *N. caninum*

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Chapter 4

Protective effect of intranasal immunization with *Neospora caninum* membrane antigens against murine neosporosis established through the gastrointestinal tract

Protective effect of intranasal immunization with *Neospora caninum* membrane antigens against murine neosporosis established through the gastrointestinal tract

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ABSTRACT

Neospora caninum is an Apicomplexa parasite that in the last two decades was acknowledged as a main pathogenic agent responsible for economic losses in cattle industry. In the present study, the effectiveness of intranasal immunization with *N. caninum* membrane antigens plus CpG adjuvant was assessed in a murine model of intragastrically established neosporosis. Immunized mice presented a lower parasitic burden in the brain upon infection with 5×10^7 tachyzoites showing that significant protection was achieved by this immunization strategy. Intestinal IgA antibodies raised by immunization markedly agglutinated live *N. caninum* tachyzoites whereas previous opsonization with IgG antibodies purified from immunized mice sera reduced parasite survival within macrophage cells. Although an IgG1/IgG2a ratio < 1 was detected in the immunized mice before and after infection, indicative of a predominant Th1-type immune response, no increased production of interferon-γ was detected in the spleen or mesenteric lymph nodes of the immunized mice. Altogether, these results show that mucosal immunization with *N. caninum* membrane proteins plus CpG adjuvant protect against intragastrically established neosporosis and indicate that parasite-specific mucosal and circulating antibodies have a protective role in against *N. caninum* infection established through the gastrointestinal tract.

INTRODUCTION

Neospora caninum is an Apicomplexa parasite initially described as the causative agent of neuromuscular disease in dogs[1]. Although canids have been identified as *N. caninum* definitive hosts, this parasite can infect a wide range of intermediate hosts including cattle[2,3]. Infected cattle display an increase likelihood of abortion[4-10] which altogether with the high efficiency of vertical transmission[7,11-16] makes neosporosis responsible for severe economic losses[17]. Therefore, effective control methods that could prevent the parasite spread are necessary. Lack of intervention is too risky and a test and cull approach despite its effectiveness is too expensive[18]. Moreover, coccidiostatic treatment also appears as an expensive option that still raises concerns regarding its usage in animals for human consumption[18]. Vaccination appears thus as the best approach to effectively control neosporosis[18,19]. However, no commercial vaccine is currently available for neosporosis upon the recent withdrawal of Bovilis® Neoguard that nevertheless had a limited efficacy[20,21]. Therefore, development of a novel vaccine that could prevent this parasitic disease is a pressing necessity.

Attenuated *N. caninum* tachyzoites were successfully used to immunize mice[22-24] or cattle[25] against neosporosis. However, the use of attenuated strains is undesirable due to a low shelf life[26] and the possible regression to a more virulent status[19]. On the other hand, although it was reported that immunization with whole parasite lysates could protect mice from *N. caninum* infection or vertical transmission[27-32], other studies showed that immunization by using parasite lysates conferred little protection or even exacerbate the outcome of murine infection[33-35] and failed to prevent vertical transmission in cattle[36]. Recombinant *N. caninum* proteins have also been tested as potential vaccine candidates with promising although variable efficacy[37-43]. Nonetheless, and despite the fact that the gastrointestinal mucosa is a natural infection route for *N. caninum*, mucosal (intranasal) immunization against neosporosis has only been attempted in a limited number of studies which yield encouraging results[44-46]. Nevertheless, despite the immunization route used therein, the possible immune response elicited in the mucosa or associated lymphoid tissue of immunized mice was not specifically addressed.

Here, our previous described model of *N. caninum* infection established by the GI tract[47,48] was used to assess the protective effect against neosporosis of intranasal (i.n.) immunization by using *N. caninum* membrane proteins (NcMP) as target antigens. Our results show that immunization with NcMP plus CpG adjuvant conferred protection against the parasite infection. Moreover, by showing an *in vitro* effector function of mucosal and circulating antibodies, we provide evidence for a protective role of the humoral immune response against neosporosis in our model.

MATERIALS AND METHODS

Animals

Seven week-old female C57BL/6 mice were purchased to Charles River (Barcelona, Spain). Animals were kept at Instituto de Ciências Biomedicas Abel Salazar (ICBAS) animal facility throughout all the experimental procedures in specific-pathogen free conditions. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92). Authorization for the experiments was issued by the competent national board (Direcção Geral de Veterinária, document 0420/000/000/2008).

Parasites

N. caninum tachyzoites (Nc1 isolate) were kept by serial passages in VERO cells cultures, maintained in Minimal Essential Medium containing Earle's salts (Sigma, St. Louis, USA), supplemented with 10% fetal calf serum (PAA laboratories, Austria), L-Glutamine (2 mM), Penicillin (200 IU/ml) and Streptomycin (200 µg/ml) (all from Sigma), in a humidified atmosphere with 5% CO_2 at 37 ^{0}C. Tachyzoites were maintained until destruction of 80% of the host cell monolayer and were isolated as previously described[47]. Briefly, free parasites and adherent cells were recovered using a cell scraper and centrifuged at 1 500 × g for 15 min. The pellet was passed through a 25 G needle and then washed three times in PBS by centrifugation at 1 500 × g for 15 min. The resulting pellet was resuspended and passed through a PD-10 desalting column, containing Sephadex™ G-25M (GE Healthcare, Freiburg, Germany). Tachyzoites concentration was determined in a haemocytometer.

Preparation of whole tachyzoite lysates and cell-membrane extracts

N. caninum membrane proteins (NcMP) were extracted using a modification of a previously described method[49,50]. Briefly, free tachyzoites were resuspended in

PBS containing 0.75% triton X-114 (Sigma), incubated 10 min on ice and centrifuged at 10 000 × g for 30 min at 4 ^{0}C and the supernatant was recovered and placed in a water bath at 30 ^{0}C during 3 min. The procedure was repeated and the supernatant was centrifuged at 1 000 × g for 3 min at room temperature. The aqueous phase was discarded and the NcMP were precipitated with the addition of absolute ethanol, vortexed vigorously during 15 sec and incubated 1 h on ice. The samples were centrifuged at 12 000 × g for 20 min at 4 ^{0}C and the resulting pellet was dried, resuspended in PBS and stored at -20 ^{0}C.

Whole *N. caninum* lysates were prepared by disruption of tachyzoites following sonication (20 cycles of 15 s at 100 W) with a Branson cell disrupter model W 185 D in an ice bath. The obtained *N. caninum* sonicates (NcS) were sequentially passed through 0.45 and 0.2 µm pore-size filters and stored at -20 ^{0}C. Quantification of NcMP or NcS was performed by using the Lowry protein assay.

Electrophoretic analysis and western blot transfer of NcMP and NcS

Evaluation of protein migration profile in each extraction was performed using a sodium dodecyl sulphate polyacrylamide gel electrophoresis. Briefly a discontinuous (4-10% acrylamide) gel was loaded with 10 µg of NcMP or NcS, previously heated at 95 ^{0}C during 5 min, and electrophoresis was carried out at a 25 mA constant current. Protein migration profiles were visualized by using silver nitrate staining. Western blot analysis of NcMP and NcS protein fractions was performed by transfer to an activated polyvinylidene difluoride membrane (GE Healthcare) the previously separated proteins by gel electrophoresis under constant current of 60 mA.

Immunizations and tissue sample collections

Eight week-old female mice were used in 3 independent experiments with random distribution into 4 groups per experiment. Mice were immunized intranasally (i.n.) at day zero under light isoflurane anesthesia with 20 µl of: PBS containing 30 µg of NcMP (NcMP group, n=13) or 30 µg of NcMP plus 10 µg of CpG ODN 1826 VacciGrade (invivogen, San Diego, CA) (NcMP/CpG group, n=14). Sham-immunized control mice were immunized with PBS alone (PBS group, n=14) or

PBS containing, 10 µg of CpG ODN 1826 VacciGrade (CpG group, n=13). The immunization procedure was repeated 3 weeks upon the first immunization. All mice were intragastrically (i.g.) challenged, 3 weeks after the last immunization with 5 × 10^7 freshly isolated *N. caninum* tachyzoites as previously described[47]. One week after infection, mice were sacrificed by cervical dislocation and spleens and mesenteric lymph nodes (MLN) were aseptically removed for the analysis of the immune response, while the brains were collected and stored at -20 ^{0}C for DNA extraction. One week after boost immunization and 1 week after infection serum from all mice was collected in the submandibular vein for detection of *N. caninum*-specific IgG. At the same time-points, vaginal and intestinal lavages fluids (VLF and ILF, respectively) were collected respectively, for detection of *N. caninum*-specific IgA.

Antibody detection

Serum IgG1 and IgG2a titres specific for NcMP were quantified by ELISA. Briefly, 96-well plates (Maxisorp, Nunc, Denmark), were coated overnight at 4 ^{0}C with NcMP diluted in PBS at a concentration of 5 µg/ml. All the wells were saturated with 2% bovine serum albumin (BSA) (Sigma) in TST buffer (150 mM NaCl, 10 mM EDTA and 0.05% Tween 20, pH=8) for 1 h. Serum samples were serial diluted in 1% BSA TST buffer and incubated for 1 h, followed by washing and addition of alkaline phospatase-coupled monoclonal goat anti-mouse IgG1 or IgG2a monoclonal antibodies (Southern Biotechnology Associates, Birmingham, USA) and incubation for 1 h. After washing, the specifically bound antibodies were detected by the development with the substrate solution of p-nitrophenyl phosphate (Sigma) and the reaction was stopped with the addition of 0.1 M EDTA, pH=8 solution. The absorbance was measured at 405 nm, subtracting for each well the value for the absorbance at 570 nm. The antibody titres were expressed as the $\log_{10}$ value of the reciprocal highest dilution with an absorbance higher than the value of the control (no serum added). Detection of IgA antibodies specific for NcMP were quantified by ELISA as previously described, using alkaline phospatase-coupled goat anti-mouse anti IgA mAb (Southern Biotech).

Purification of serum IgG antibodies and mucosal IgA

Mice serum samples and ILF collected at the day mice euthanasia were respectively used for IgG and IgA purification. Pooled sera of mice from the NcMP/CpG and CpG groups were used to purify IgG antibodies by using a HiTrap Protein G HP purification column (GE healthcare), according to manufacturer's instructions. Recovered antibodies were buffer exchanged against sterile PBS to a final concentration of 4.5 mg/ml as determined by Lowry protein assay and stored at -20 ^{0}C. The purified IgG fractions obtained from the sera of CpG or NcMP/CpG mice groups were respectively designated as IgG-CpG or IgG-NcMP/CpG. The NcMP-specific antibody titre of the IgG-CpG and IgG-NcMP/CpG preparations were below detection limit and 1.559×10^{9}, respectively, as determined by ELISA.

To obtain purified IgA antibodies, pooled ILF were passed through a 0.2 µm pore-size filter before being introduced in a Protein L/Agarose (Invivogen) column. Antibody purification was carried out according to manufacturer's instructions. Recovered antibodies were buffer exchanged against sterile PBS, and stored at -20 ^{0}C. The purified IgA fractions obtained from the ILF of CpG or NcMP/CpG mice groups were respectively designated as IgA-CpG or IgA-NcMP/CpG. The total IgA titres for the IgA-CpG and IgA-NcMP/CpG preparations were of 657648 and of 786788, respectively, and both fractions were normalized to 650×10^{3} for further usage. The NcMP-specific IgA titres of the IgA-CpG and IgA-NcMP/CpG preparations were below detection limit and 8995, respectively.

Western blot analysis

Western blot membranes containing NcMP and NcS protein extracts were used to determine the immunoreactivity of the purified IgG-NcMP/CpG and IgA-NcMP/CpG fractions and confirm the existence of immunodominant antigens present in each protein extract. Briefly, membranes were saturated with 1% BSA (Sigma) in TST buffer for 2 h. IgG-NcMP/CpG and IgA-NcMP/CpG antibody fractions were incubated with the membranes at a dilution of 1:2000 and 1:1000, respectively for 1 h in 0.1% BSA TST, followed by washing and addition of either alkaline phospatase-coupled monoclonal goat anti-mouse IgG1 or IgG2a or IgA monoclonal antibodies (Southern Biotech) at a dilution of 1:2000. After washing,

the specifically bound antibodies were detected by the development with the substrate solution of NBT/BCIP (Roche, Basel, Switzerland) and the reaction was stopped by washing the membrane with deionized water.

Antibody-binding and parasite-agglutination assays.

To evaluate the ability of antibodies present in IgG- or IgA-CpG, or IgG- or IgA-NcMP/CpG fractions to bind *N. caninum*, different dilutions of these preparations were incubated with 1×10^6 tachyzoites, for 25 min on ice. Detection of bound antibodies was made using flow cytometry, for which parasites were further incubated with polyclonal anti-IgG antiserum, fluorescein isothiocyanate (FITC) conjugate (Southern Biotech), or with anti IgA-FITC conjugate (BDbiosciences, San Diego, USA) mAb (clone C10-3) for 25 min on ice and then washed with PBS containing 1% BSA and 10mM sodium azide (FACS buffer). Parasite samples were analyzed in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter, MI). The collected data files (100,000 events per sample) were converted for analysis with the Cell Quest software, v3.2.1f1 by using FACS convert, v1.0 (both from Becton Dickinson, San Jose, USA). Agglutination assays were performed by incubating 1×10^6 tachyzoites with either IgA-CpG or IgA-NcMP/CpG or PBS alone 1h at 4 ºC. After incubation smears of each sample were prepared on microscopic slides that were fixed in cold methanol for 5 min. Samples were then stained with Hemacolor 2 and 3 (Merk, Darmstadt, Germany) according to manufacturer's instructions. Mounted slides were observed in a light microscope and 20 pictures at 20× magnification were taken (Leica Qwin plus v3.5.1) as a representative display of each slide. Analysis of the number and size of parasite clusters were performed by using ImageJ software (Version 1.47, National Institutes of Health, Bethesda, MD)

Intracytoplasmic staining

For intracellular cytokine detection by flow cytometry, spleens and MLN were aseptically removed from the euthanized infected mice, homogenised in HBSS (Sigma) and red blood cells were lysed. The remaining cells were counted and plated in round bottom 96-well plates (Nunc), at a concentration of 1×10^6 cells/ml

in RMPI-1640 (Sigma) supplemented with 10% fetal calf serum (PAA laboratories), HEPES (10 mM), Penicillin (200 IU/ml) and Streptomycin (200 µg/ml) (all from Sigma), β-mercaptoethanol (0.1 µM) (Merk) (RPMI complete medium). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 ^{0}C for 4.5 h under stimulation with 20 ng/ml phorbol myristate acetate (Sigma) and 200 ng/ml ionomycin (Merk) and 10 ng/ml of Brefeldin A (Epicentre Biotechologies, Madison, USA). Then, cells were recovered and unspecific antibody binding was prevented by the pre-incubation with anti-FcγR mAb followed by incubation with either anti-CD4 Peridinin-chlorophyll proteins-cychrome 5.5 (PerCP-Cy5.5)-conjugate (clone RM4-5) or anti-CD8 PerCP-Cy5.5-conjugate (clone 53-6.7) mAb (both from BDbiosciences). Following extracellular staining the cells were washed, fixed in 2% formaldehyde, washed again and permeabilized with 0.05% saponin (Sigma) PBS solution and intracytoplasmic staining was carried out with anti-IFN-γ FITC-conjugate (clone XMG1.2), anti-IL-4 Phycoeritrin (PE)-conjugate (clone BVD4-1D11) and anti-IL-10 PE-conjugate (clone JES5-16E3) (all from BDbiosciences) after pre-incubation of the cells with anti-FcγR mAb. Antibody-labelled cells were analyzed in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter). At least 150,000 events were acquired per sample. The collected data files were converted using FACS convert, v1.0 (Becton Dickinson) and analyzed using Cell Quest software, v3.2.1f1 (Becton Dickinson).

***In vitro* cell cultures and cytokine detection.**

To assess *in vitro* cytokine production by NcMP-stimulated spleen cells, 5 ml aliquots of cell suspensions prepared as described above for intracytoplasmic staining, were layered onto 2.5 ml of a polysucrosesodium ditrizoate solution (Histopaque 1083, Sigma) and centrifuged at 800 × g for 20 min. Mononuclear cells collected from the medium-Histopaque interface were washed, suspended in RPMI complete medium, plated (5 × 10^5/well) in round bottom 96-well plates (Nunc), and stimulated with NcMP (100 µg/ml) for 5 days at 37 ºC and 5% CO_2. Four animals from each group were used and triplicate wells were set for cells cultured from each animal. The concentration of IFN-γ and IL-4 in cell culture supernatants were respectively quantified with the Mouse IFN-γ DuoSet® ELISA development system (R&D Systems, Minneapolis, MN) and IL-4 ELISA Ready-

Set-Go!® (eBioscience, San Diego, CA) kits, both according to manufacturer's instructions.

Macrophage cell cultures and parasite opsonization survival

Mouse bone marrow-derived macrophages (BMDM) were differentiated from bone marrow precursors. The BMDM cultures were generated in 6-well plates (Nunc) by culturing 5×10^6 cells in 5 ml of RPMI complete medium supplemented with 10% of L-929 cell line conditioned medium (LCCM) and incubated at 37 ºC in a 5% CO_2 humidified chamber. On day 4, the cell culture medium was renewed and 5 ml of fresh medium supplemented with LCCM was added. Differentiated macrophages were harvested on day 7 by gently scraping the wells. The cells were counted and plated in 24-well plates (Nunc) at a concentration of 1×10^6 cells/ml. BMDM were then infected at a multiplicity of infection (MOI) of 1:1 with *N. caninum* tachyzoites incubated with IgG-NcMP/CpG or IgG-CpG, as described above, at different dilutions or untreated parasites as control. Cells were incubated for 6 h at 37 ºC in a 5% CO_2 humidified chamber.

DNA extraction

DNA from the brain of infected mice and *in vitro* BMDM cell cultures was extracted as previously described[51]. Briefly, brains were weighted and homogenized. Samples were incubated overnight at 55 ^{0}C in a solution containing 1% SDS and 1 mg/ml Proteinase K (sigma). DNA was extracted by the phenol (Sigma)-Chlorophorm (Merk) method followed by ammonium acetate/ethanol precipitation.

Real-time PCR analysis

The parasite burden in the brain of infected mice and macrophage cell cultures was assessed by a quantitative real-time PCR (qPCR) analysis of the parasite DNA performed in a Corbett rotor gene 6000 system (Corbett life science, Sydney, Australia). Brain analysis was performed by using Rotor-Gene probe PCR kit (Quiagen, Hilden, Germany), for the amplification of a 103 bp sequence of the Nc5 region of *N. caninum* genome using the primers NcA 5'

GCTACCAACTCCCTCGGTT 3' and NcS 5' GTTGCTCTGCTGACGTGTCG 3' both at a final concentration of 0.2 µM, and the florescent probe FAM-CCCGTTCACACACTATAGTCACAAACAAAA-BBQ at a final concentration of 0.1 µM (all design and obtained from TIB-Molbiol, Berlin, Germany). The DNA samples were amplified using the following program: 95 ^{0}C for 3 min, 95 ^{0}C for 5 sec, 60 ^{0}C for 20 sec with fluorescence acquisition. The second and third steps were repeated 50 times. Length of the amplified DNA was confirmed in a 3% agarose gel stained with ethidium bromide. BMDM samples were analyzed using Express Sybr green ER qPCR supermix universal (Invitrogen), for the amplification of a 337 bp sequence of the Nc5 region of *N. caninum* genome using the primers Np21plus 5' CCCAGTGCGTCCAATCCTGTAAC 3' and Np6plus 5' CTCGCCAGTCAACCTACGTCTTCT 3' (both from TIB-Molbiol), both at a final concentration of 0.25 µM. The DNA samples were amplified using the following program: 95 ^{0}C for 10 min, 95 ^{0}C for 30 sec, 63 ^{0}C for 20 sec, 72 ^{0}C for 45 sec with fluorescence acquisition, the second, third and fourth step were repeated 45 times. A melting curve was performed in each run in order to access the PCR amplified fragments: from 65 ^{0}C to 95 ^{0}C, with increments of 1 ^{0}C for 5 seconds. In all runs parasite burden was determined by interpolation of a standard curve, ranging from 10 to 10^{-4} ng of DNA extracted from *N. caninum* tachyzoites included in each run and the data analyzed in the Rotor gene 6000 software v1.7 (Corbett life science).

Statistical analysis

Statistical analyses were performed using GraphPad prism, Version 5.0 (GraphPad Software, Inc., La Jolla, CA). In the scatter dot graphs the mean for each group was displayed as a horizontal line. Column graphs are represented showing the mean plus one standard error of the mean (SEM). Mean florescence intensity (MFI) values in flow cytometry analysis are presented with mean plus one standard deviation (SD) Statistical analysis was performed by using one-way ANOVA with Newman-Keuls post-hoc analysis.

RESULTS

NcMP electrophoresis migration profile and western blot analysis

In order to determine the NcMP and NcS protein migration profile, both extracts were separated in a reducing and denaturating SDS-Page gel. As shown in Figure 4-1a, and following silver nitrate staining, the NcMP extraction protocol presented an enrichment of the proteins with approximately 55, 35 and 29 kDa as compared with the protein profile observed in NcS preparation. Additionally, the NcMP extraction protocol yielded two proteins with an estimated molecular weight of 39 and 17 kDa that could not be observed in the NcS protein migration profile.

In order to determine the existence of immunodominant antigens in both protein extracts, a western blot analysis of previously separated NcMP and NcS was performed using purified serum IgG and intestinal IgA obtained from mice i.n. immunized with NcMP and CpG. As shown in Figure 4-1b, incubation with purified serum IgG and development with either anti-IgG1 or IgG2a revealed that all previously identified NcMP protein bands reacted strongly with IgG of both isotypes. In contrast, only faint development was observed in similarly incubated and revealed NcS extracts. Additionally, a previously unobserved protein band in the silver nitrate stained gel with approximately 45 kDa also presented strong reactivity in the NcMP extracts. Similar incubation with purified intestinal IgA revealed similar results although protein reactivity with anti-IgA incubation was substantially lower. Once again, NcS incubation with purified intestinal IgA revealed only faint protein bands. Altogether these results indicate that the NcMP extraction protocol was enriched in some proteins already present in NcS fractions. They also show that proteins corresponding to major bands in NcMP extracts were immunogenic following i.n. immunization with CpG adjuvant.

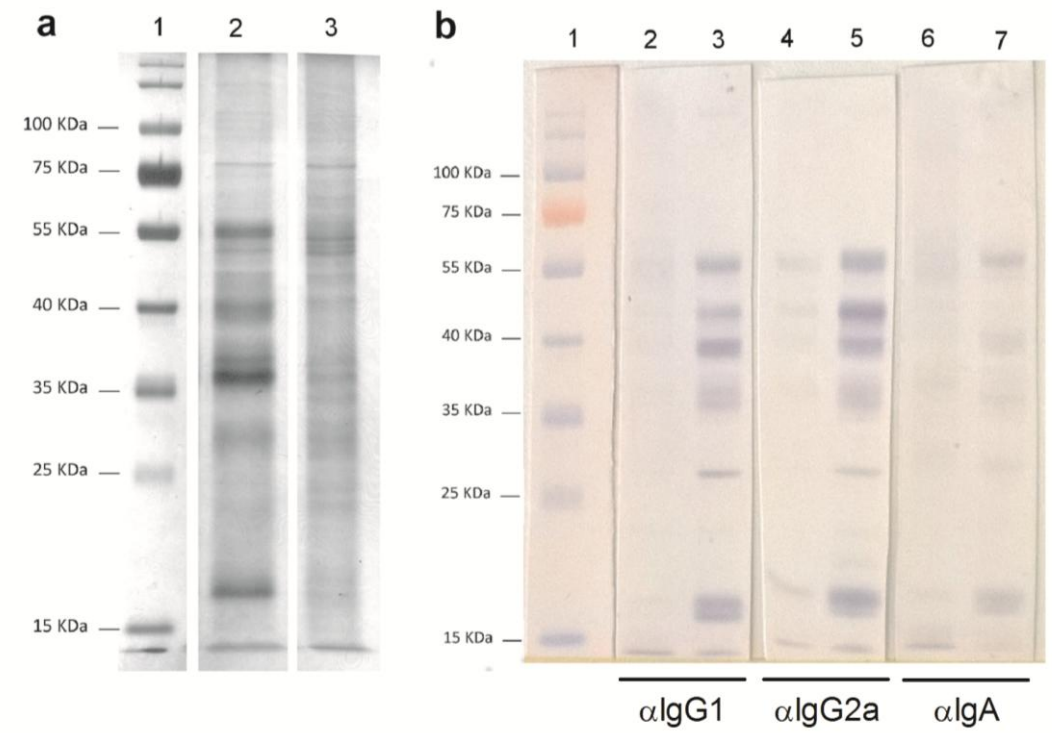


Figure 4-1 - (a) Electrophoretic migration profiles of *N. caninum* protein extracts. SDS-Page gel stained with silver nitrate, representative of the pattern of protein migration obtained for *N. caninum* membrane proteins (NcMP) (lane 2) and *N. caninum* sonicates (NcS) (lane 3). Numbers on the left correspond to the molecular weight, in kDa, of the molecular weight marker (lane 1). (b) Western blot analysis of NcMP and NcS following protein separation in a SDS-Page gel. Western blot was incubated with purified serum IgG and intestinal IgA from NcMP/CpG immunized mice and respectively revealed with anti-IgG1, IgG2a or IgA, as indicated. Molecular weight marker (lane 1), NcS (lanes 2, 4 and 6) and NcMP (lanes 3, 5 and 7).

Intestinal mucosa IgA produced upon immunization binds to and agglutinates *N. caninum* tachyzoites

Production of IgA is the hallmark of specific immune responses at the mucosa[52-55]. As IgA levels in the small intestine were difficult to ascertain without invasive procedures, NcMP-specific IgA titres were measured in VLF to monitor the effectiveness of the mucosal immunization. As shown in Figure 4-2a, higher titres of antigen-specific IgA were detected in the VLF of mice from the NcMP/CpG group than in the other groups 1 week upon the boosting i.n. immunization. Accordingly, elevated levels of NcMP-specific IgA were detected in ILF of the

NcMP/CpG mice group, as compared with the other groups studied, 1 week after infection (Figure 4-2b). Mice of the NcMP group also presented higher antibody levels in the VLF and ILF than control groups at both assessed time points, although lower than those of the NcMP/CpG group, thus highlighting the adjuvant effect of CpG.

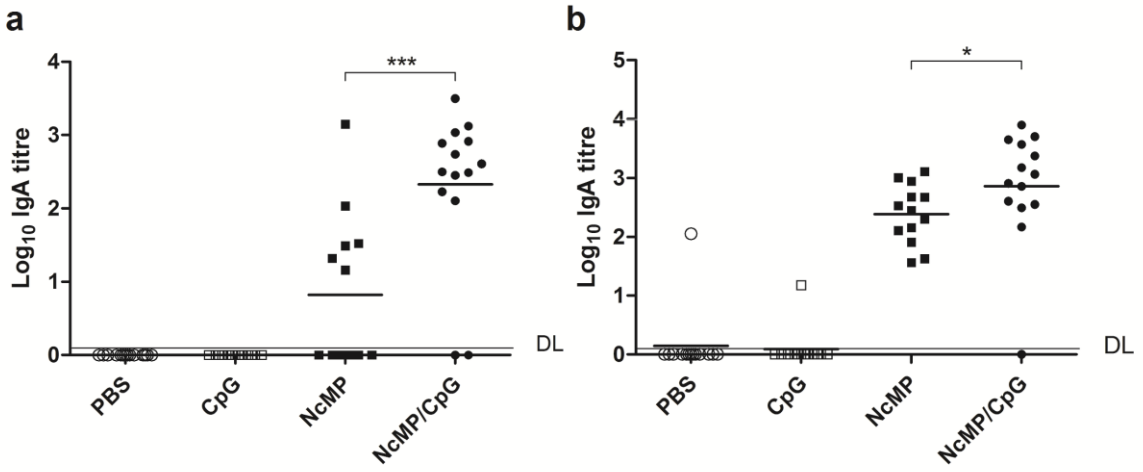


Figure 4-2 - *N. caninum* membrane proteins (NcMP)-specific IgA titres detected by ELISA in vaginal and intestinal lavage fluids (VLF and ILF, respectively). IgA titres were determined by ELISA in (a) VLF collected one week upon the second immunization from mice immunized twice i.n. with NcMP with or without CpG adjuvant (NcMP and NcMP/CpG, respectively) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG); (b) ILF collected from the same groups one week after i.g. challenged with 5×10^7 *N. caninum* tachyzoites performed 3 weeks upon the last immunizing administration. Data is presented as $\log_{10}$ of the antibody titres, as indicated. Results correspond to pooled data of three independent experiments (PBS n=14; CpG n=13; NcMP n=13; NcMP/CpG n=14). Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group; * $p<0.05$; *** $p<0.001$; detection limit (DL) is indicated by a horizontal line.

The ability of the raised IgA antibodies to bind *N. caninum* tachyzoites was confirmed using flow cytometry. As shown in Figure 4-3a, a significant increase in the MFI value due to parasite-bound IgA was observed in tachyzoites incubated with IgA-NcMP/CpG as compared to that of parasites incubated with IgA-CpG, thus confirming the specificity of the antibodies raised by immunization. The agglutination of pathogens is one recognized effector function of IgA in the

intestinal mucosa, thus preventing their attachment to host cells[56]. To assess the agglutinating capacity of the intestinal IgA produced upon i.n. immunization, *N. caninum* tachyzoites were incubated with IgA-NcMP/CpG and IgA-CpG and the formation of parasitic agglutinates assessed by optical microscopy. Upon incubation, a higher number of parasite clusters and a higher area per cluster (Figure 4-3b and c, respectively) were found when the tachyzoites were incubated with IgA-NcMP/CpG than with IgA-CpG or PBS alone. Parasites incubated with IgA-CpG or PBS were mainly observed as single cells, while parasites treated with IgA-NcMP/CpG were mainly observed agglutinated in large bodies (Figure 4-3d). Altogether, these results show that mucosal parasite-agglutinating IgA antibodies are produced upon i.n. immunization targeting NcMP.

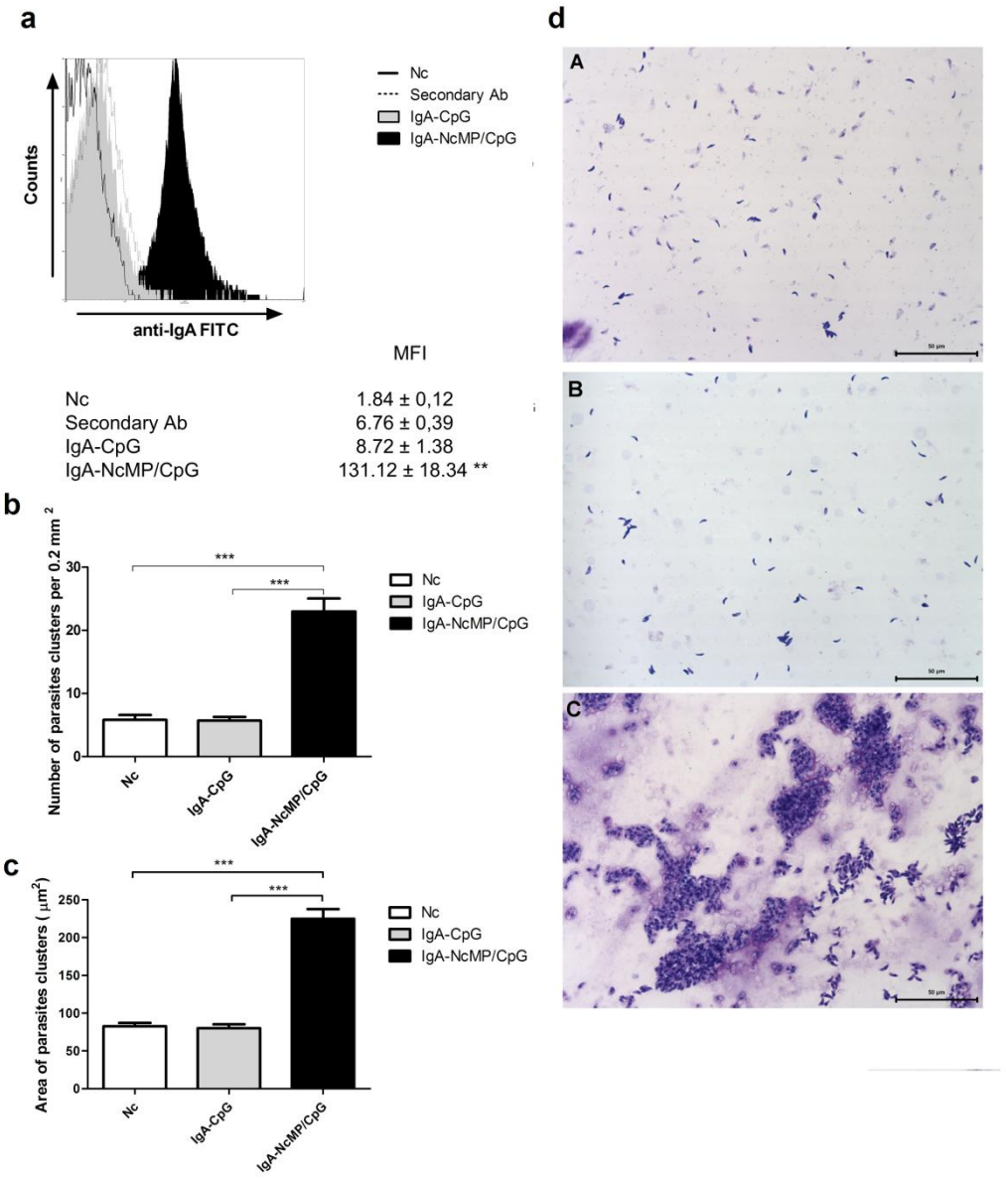


Figure 4-3 - (a) Flow cytometric analysis of anti-IgA-FITC staining of *N. caninum* tachyzoites incubated with intestinal IgA antibodies collected from mice one week upon i.g. infection with 5×10^7 *N. caninum* tachyzoites 3 weeks upon the second of two i.n. immunizations with *N. caninum* membrane proteins plus CpG adjuvant (IgA-NcMP/CpG) or similarly treated with CpG alone (IgA-CpG), or with anti-IgA FITC alone (Secondary Ab), or untreated (Nc). Histograms are of a representative example from 3 independent experiments with n=3 for each condition. The mean fluorescence intensity (MFI) ± one SD is indicated. Statistical significance of the IgA-NcMP/CpG condition as compared with any of the other conditions is indicated (** $p<0.01$). Parasite agglutination was assessed by Hemacolor staining of tachyzoites incubated with PBS (Nc) or IgA-CpG or IgA-NcMP/CpG fractions for 1 h at 4 ºC. Analysis of the number (b) and size (c) of parasite clusters was made with pooled results of 20 micrographs taken from each condition at a 200× magnification. Parasite clustering was considered for 4 or more parasites appearing bound together. Bars correspond to tachyzoites incubated in: PBS alone (Nc), IgA-CpG alone (IgA-CpG) or with IgA-NcMP/CpG (IgA-NcMP/CpG), as indicated. Results are of one representative example out of three independent experiments. Each bar represents the mean value for each group. Error bar = SEM (*** $p<0.001$). (d) Representative microscopic analysis of *N. caninum* tachyzoites clustering assessed by Hemacolor staining. Tachyzoites were incubated with (A) PBS, (B) IgA-CpG or (C) IgA-NcMP/CpG fractions. Micrographs for each condition were obtained at 400× magnification.

Serum IgG antibodies elicited upon immunization with NcNP plus CpG bind to *N. caninum* and reduce parasite survival in infected macrophages

To determine whether parasite-specific IgG antibodies were also induced by the i.n. immunization, serum samples were analyzed for the presence of NcMP-specific antibodies of that isotype. As shown in Figure 4-4, the majority of the immunized mice presented high levels of antigen-specific IgG antibodies, detected prior to or after the i.g. parasitic challenge. All but one mice i.n. treated with CpG or PBS alone presented detectable serum IgG antibodies with this specificity by 1 week upon the parasitic challenge. Analysis of the IgG isotype profile revealed a mixed IgG1 and IgG2a response in the NcMP and NcMP/CpG groups. However, disparate IgG1/IgG2a ratios were detected in these groups. While in mice immunized with NcMP alone, this ratio was > 1 prior or after infection, a ratio < 1 was observed for the NcMP/CpG group (Figure 4-4). As the IgG2a and IgG1

isotypes were respectively associated to a Th1 and a Th2-type immune response[57], these results indicate that a predominant Th1-type immune response was induced in the NcMP/CpG group while a Th2-type immune response was elicited by NcMP immunization in the absence of adjuvant.

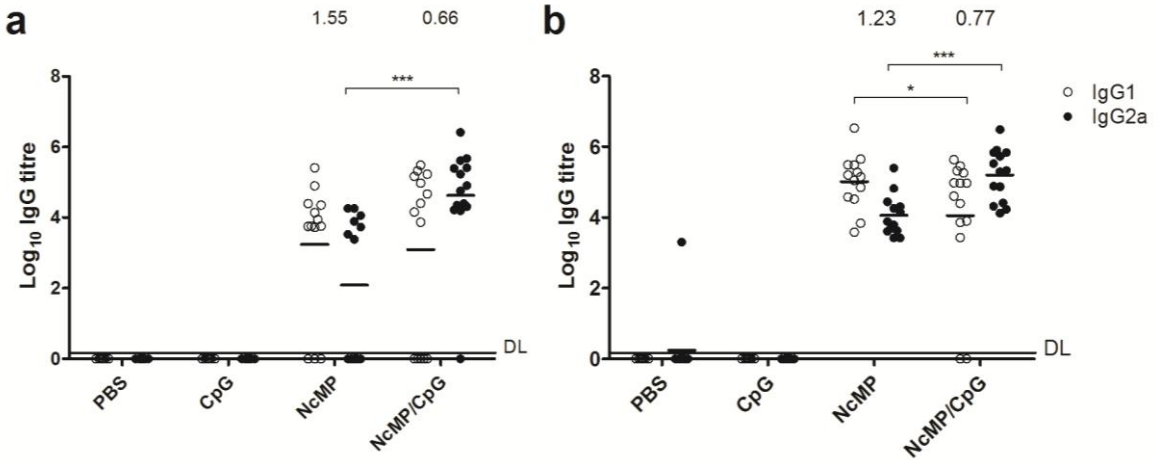


Figure 4-4 - Titres of *N. caninum* membrane proteins (NcMP)-specific serum IgG1 (open circles) and IgG2a (closed circles) antibodies determined by ELISA (a) one week upon the second immunization in mice immunized twice i.n. with NcMP with or without CpG adjuvant (NcMP and NcMP/CpG, respectively) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG) or (b) in the same groups, one week after an i.g. challenge with 5×10^7 *N. caninum* tachyzoites performed 3 weeks upon the last immunizing administration. Data is presented as $\log_{10}$ of the antibody titres, as indicated .Numbers above each group represent the IgG1/IgG2a ratio, calculated with the mean $\log_{10}$ titres for the correspondent IgG isotype. Results correspond to pooled data of three independent experiments (PBS n=14; CpG n=13; NcMP n=13; NcMP/CpG n=14). Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group (* $p<0.05$; *** $p<0.001$; detection limit (DL) is indicated by a horizontal line.

To determine the ability of serum IgG produced in the immunized mice to bind *N. caninum* parasites, tachyzoites were incubated with either IgG-NcMP/CpG or IgG-CpG and analyzed by flow cytometry. As shown in Figure 4-5a, IgG-NcMP/CpG antibodies bound *N. caninum* tachyzoites more markedly than those in the IgG-CpG preparation.

N. caninum is an obligate intracellular parasite and therefore the capacity to infect new cells once inside the host is essential for its survival. Therefore, blocking the infection of new cells could be an important factor for parasite control. In order to test the effects of the IgG preparations in the capacity of *N. caninum* to survive in BMDM cell cultures, tachyzoites were incubated with the IgG antibody preparations. The opsonized tachyzoites as well as non-opsonized counterparts were used to challenge macrophage cell cultures for 6 h upon which the number of parasites therein was evaluated by qPCR. As shown in Figure 4-5b, parasite opsonization with IgG raised by immunization resulted in a dose-dependent reduction in the total number of parasites detected in the cultures.

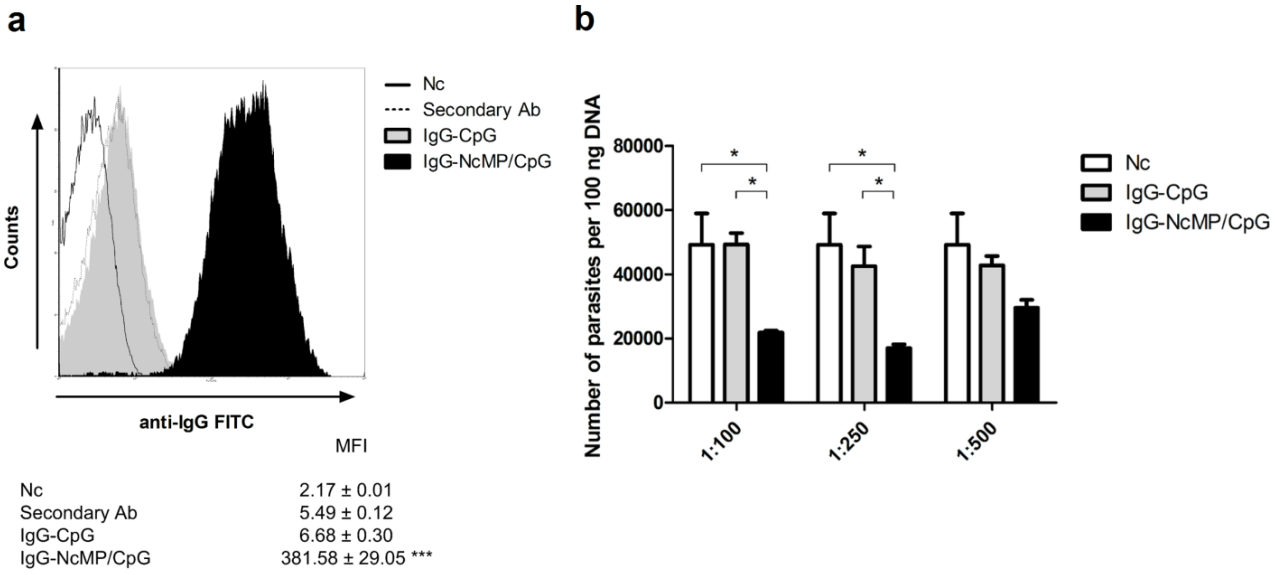


Figure 4-5 - (a) Flow cytometric analysis of anti-IgG-FITC polyclonal antibody staining of *N. caninum* tachyzoites incubated with serum IgG antibodies collected from mice one week upon i.g. infected with 5×10^7 *N. caninum* tachyzoites 3 weeks upon immunized twice i.n. with *N. caninum* membrane proteins plus CpG adjuvant (IgG NcMP/CpG) or similarly treated with CpG alone (IgG-CpG), or with anti-IgG-FITC alone (Secondary Ab), or untreated (Nc). Histograms are a representative example from one out of 3 independent experiments (n=3 for each condition). The mean fluorescence intensity (MFI) ± one SD is indicated. Statistical significance of the IgG-NcMP/CpG condition as compared with any of the other conditions is indicated (*** $p<0.001$). (b) Number of parasites, assessed by qPCR in bone marrow-derived macrophage cell cultures challenged at a MOI of 1:1 for 6h with 1×10^6 tachyzoites previously incubated with IgG-CpG or IgG-NcMP/CpG at the indicated dilutions, or untreated parasites (Nc). Results are of a representative example out of 3 independent experiments. Each bar represents the mean of 3 wells. Error bar = SEM (* $p<0.05$)

Protective effect of mucosal immunization against i.g. established neosporosis.

Neosporosis is thought to be horizontally transmitted by oocyst ingestion in naturally infected hosts[3]. Therefore, it is conceivable that boosting the immune response in the gastrointestinal mucosa by parasite-specific immunization may increase host resistance against this parasitic disease. To assess whether the immunization procedures used here could be protective, we evaluated by using qPCR the parasitic burden in the brain of mice of the different groups, one week upon i.g. administration of 5×10^7 *N. caninum* tachyzoites performed three weeks upon the last immunization. As shown in Figure 4-6, a significant reduction of the mean parasitic DNA level in mice of the NcMP/CpG group was detected, as compared with that in control mice, which received CpG or PBS alone. Moreover, the NcMP/CpG group was the one showing the highest number of mice with absent or below detection-limit parasitic DNA (n=4; n=5 and n=10 in the PBS, CpG and NcMP, and NcMP/CpG groups, respectively). Interestingly, the mouse within the NcMP/CpG group showing higher parasitic colonization was the only that did not present detectable levels of IgA in either VLF or ILF, as shown in Figure 4-2. No significant differences were found among any other groups, although the mean parasitic burden detected in the NcMP/CpG group was greatly reduced as compared to that of the NcMP group. These results altogether show that i.n. immunization with NcMP plus CpG adjuvant confers protection against neosporosis established by the gastrointestinal tract.

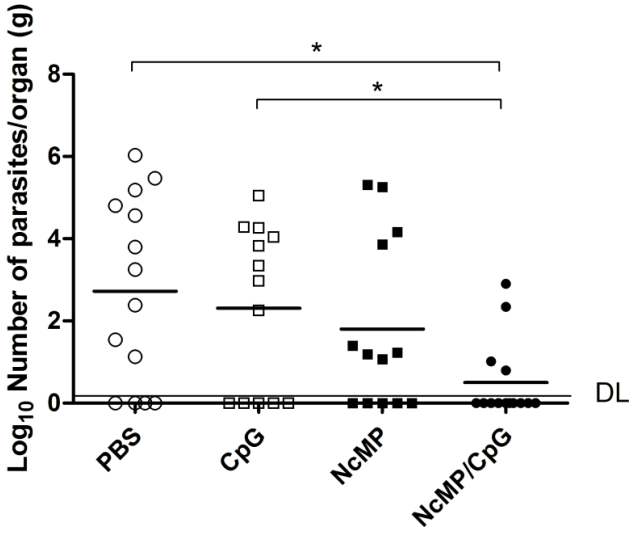
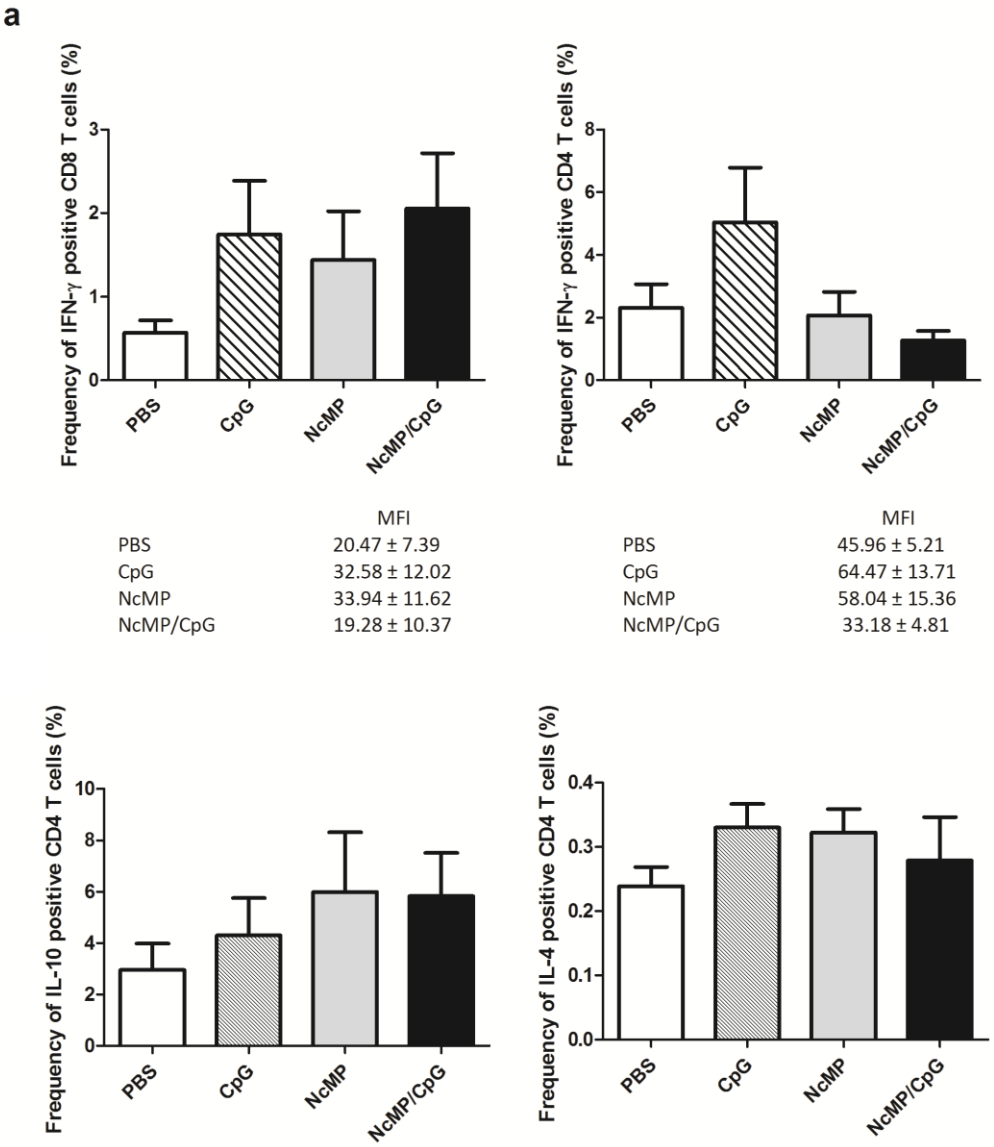


Figure 4-6 - Parasitic load assessed by qPCR one week upon i.g. challenge with 5×10^7 *N. caninum* tachyzoites in the brain of mice previously immunized twice i.n. with *N. caninum* membrane proteins with or without CpG adjuvant (NcMP and NcMP/CpG, respectively) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG). Results are from pooled data of three independent experiments. (PBS n=14; CpG n=13; NcMP n=13; NcMP/CpG n=14). Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group (* $p<0.05$); detection limit (DL) is indicated by a horizontal line.

Cytokine production in the immunized mice

Production of IFN-γ is associated with host resistance to neosporosis whereas production of IL-4 and IL-10 associate with host susceptibility to this infection[58,59]. Therefore, the frequency and numbers of cells producing these cytokines were assessed in the spleen and MLN of immunized mice and controls one week upon i.g. infection. Unexpectedly, no differences were observed among the different analyzed groups in the frequency of splenic $CD4^+$ T cells producing any of these cytokines (Figure 4-7a). Similarly, no significant difference in the frequency of splenic IFN-γ-producing $CD8^+$ T cells was observed among groups (Figure 4-7a). In order to determine whether, despite present in similar frequencies to those of controls, $CD4^+$IFN-γ^+ and $CD8^+$IFN-γ^+ T cells of immunized mice could be producing higher amounts of this cytokine the MFI due to the IFN-γ staining was assessed in these lymphocyte populations. Results shown in Figure 4-7a indicate that such is not the case, as MFI values detected among all assessed groups did not significantly very. Nevertheless, and surprisingly, lower numbers of splenic $CD4^+$IFN-γ^+ cells were observed in the NcMP/CpG group as compared with the respective CpG control group (Figure 4-7b). Similarly, lower numbers of $CD8^+$IFN-γ^+ cells were also observed in the NcMP/CpG group, although not reaching statistical significance (Figure 4-7b). Furthermore, no significant differences between all analyzed groups were observed regarding the number of $CD4^+$ T cells producing IL-10 and IL-4 (Figure 4-7b). The NcMP/CpG group also presented significantly lower numbers of total $CD4^+$ and $CD8^+$ T cells, as compared with the CpG and NcMP groups (Figure 4-7c). Similar analyses to all those performed in the spleen were also done in the MLN that did not show any

significant difference among mice groups (data not shown). Moreover, no significant differences among groups were observed in the levels of IFN-γ or of IL-4 detected in cell culture supernatants of NcMP-stimulated splenocytes collected from the different analyzed mice, week upon infection (Figure 4-7d). Cytokine levels in similarly stimulated MLN cell cultures were consistently found near or below detection limits (data not shown). Altogether, these results indicate that in the NcMP/CpG group, the cellular immune response elicited upon the parasitic challenge was comparable to that of non-immunized PBS-treated infected mice and less noticeable than in the mice that received CpG adjuvant or NcMP alone.



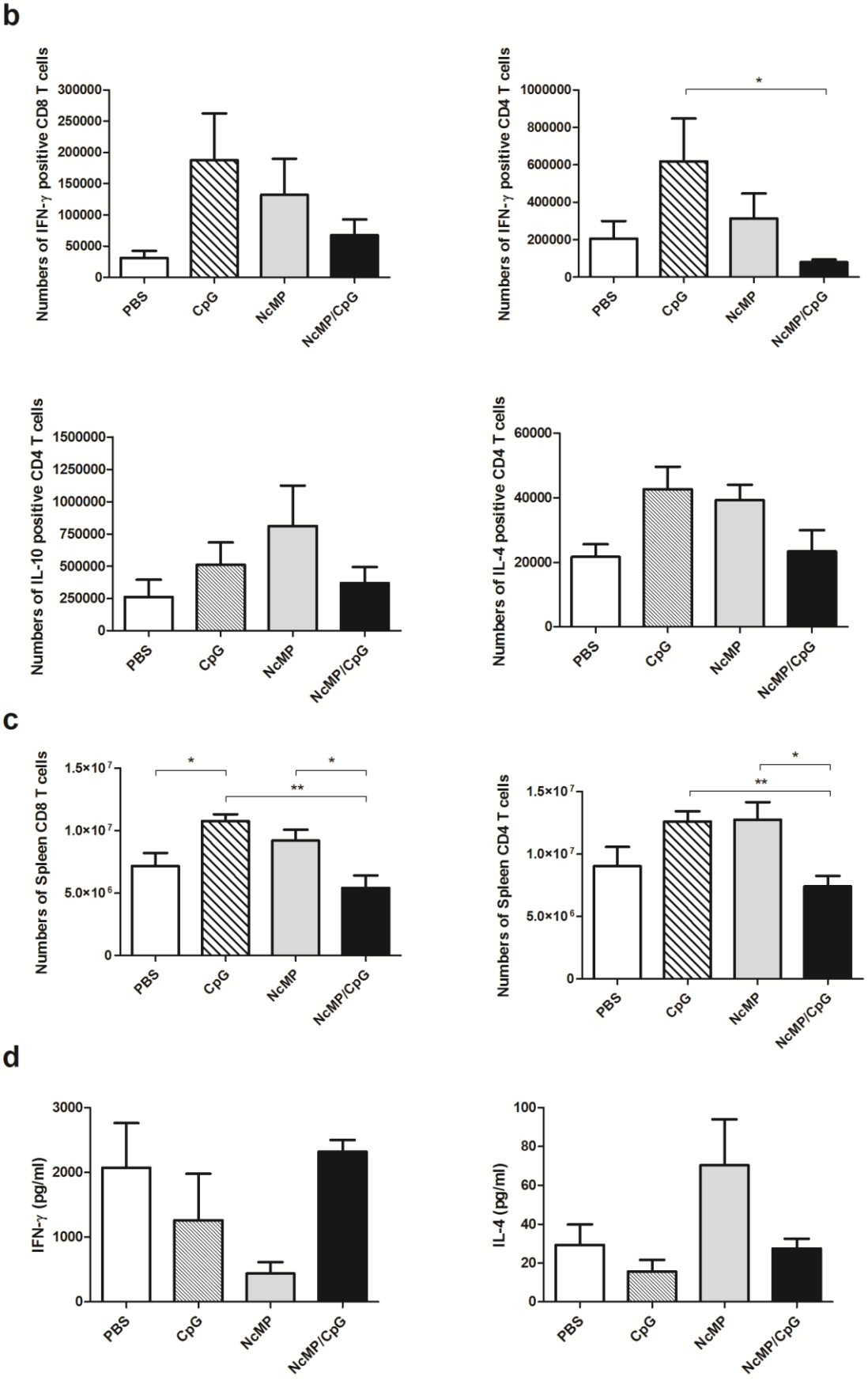

b
Numbers of IFN-γ positive CD8 T cells
300000
250000
200000
150000
100000
50000
0
PBS
CpG
NcMP
NcMP/CpG
Numbers of IFN-γ positive CD4 T cells
1000000
800000
600000
400000
200000
0
*
PBS
CpG
NcMP
NcMP/CpG
Numbers of IL-10 positive CD4 T cells
1500000
1250000
1000000
750000
500000
250000
0
PBS
CpG
NcMP
NcMP/CpG
Numbers of IL-4 positive CD4 T cells
60000
40000
20000
0
PBS
CpG
NcMP
NcMP/CpG
c
Numbers of Spleen CD8 T cells
1.5×10^7
1.0×10^7
5.0×10^6
0
*
*
**
PBS
CpG
NcMP
NcMP/CpG
Numbers of Spleen CD4 T cells
1.5×10^7
1.0×10^7
5.0×10^6
0
*
**
PBS
CpG
NcMP
NcMP/CpG
d
IFN-γ (pg/ml)
3000
2000
1000
0
PBS
CpG
NcMP
NcMP/CpG
IL-4 (pg/ml)
100
80
60
40
20
0
PBS
CpG
NcMP
NcMP/CpG

Figure 4-7 - Frequency (a) and total numbers (b) of splenic IFN-γ+ or IL-4^+ or IL-10^+ $CD4^+$ or IFN-γ^+CD8^+ T cells, or of (c) total splenic $CD8^+$ and $CD4^+$ T cells, detected one week after i.g. challenge with 5×10^7 *N. caninum* tachyzoites in mice previously immunized with *N. caninum* membrane proteins with or without CpG adjuvant (NcMP and NcMP/CpG, respectively) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG). The mean fluorescence intensity (MFI) ± one SEM due to IFN-γ staining is indicated for each group for both $CD4^+$ and $CD8^+$ T cells in (a). Results correspond to pooled data of two independent experiments. PBS, CpG and NcMP n=8; NcMP/CpG n=9. Each bar represents the mean value for each group. Error bar = SEM (* $p<0.05$; ** $p<0.01$). (d) IFN-γ and IL-4 concentrations detected in the supernatant of NcMP-stimulated (100 μg/ml) 5-day cultures of spleen mononuclear cells collected from mice of the above referred groups and at the same post-infection time. Results are of a representative example out of 3 independent experiments with four mice per group. Triplicate wells were set per animal. Bars represents the mean value for each group. Error bar = SEM

DISCUSSION

In this study we have evaluated the host protective effect of i.n. immunization using NcMP and CpG adjuvant against i.g. established murine neosporosis. Since in horizontal transmitted neosporosis infection is established through the intestinal mucosa[60], the potentiation of a specific immune response at this site could be one of the key factors to confer protection against this parasite. In this sense, previous studies by our group have shown that following mice i.g. infection with *N. caninum* this elicits an intestinal mucosa immune response marked by an early production of IL-12 and IFN-γ[48] (Chapter 3). Regarding the use of NcMP as the target antigens in this immunization protocol, early works that determined immunodominant antigens of *N. caninum* tachyzoites have identified proteins with the exact or very similar molecular weights to the ones observed in NcMP extracts to be the most prominent antigens recognized by serum of different experimentally and naturally infected animals[49,61-63]. In addition, these proteins were found to be mainly associated with the membrane or secretory organelles. In this regard, several studies have shown that antibodies directed to several of these proteins were capable of preventing *N. caninum* invasion of new host cells[64-66], making them valued target antigens for immunization protocols. In the particular case of the NcMP extraction, a clear enrichment in several proteins and the isolation of previously unobserved proteins in NcS was achieved, as shown by the protein gel and western blot analysis clearly distinguishing our antigenic composition from others used in previous studies. Still, future work in this area is needed in order to fully characterize and identified the exact proteins used herein.

Regarding the host protective capacity of our immunization strategy, in this study we shown that mice i.n. immunized with NcMP plus CpG adjuvant presented a lower parasitic burden in the brain when infected i.g. with *N. caninum* tachyzoites than controls receiving CpG or PBS alone, proving the host protective effect of this protocol. Although previous works have indicated the potential of i.n. immunization to confer protection in *N. caninum* intraperitoneally infected mice[44-46], our data extend the protective effect of i.n. immunization to *N. caninum* infection established through the gastrointestinal tract, a route more closely resembling the one naturally followed by parasite in horizontally transmitted neosporosis. A higher

number of mice without detectable parasite DNA were observed in the NcMP/CpG group than in the other studied groups. This result might indicate that the immunization procedure used here might completely prevent parasite colonization in some of the infected mice. Nevertheless, further experiments will be necessary to more rigorously determine to what extent this may have happened as some of the mice that received CpG or PBS alone also presented parasite DNA below the detection limit. In this regard, absence of detectable colonization in a small percentage of mice following i.g. infection have previously been reported, which could account for the lower number of undetectable parasitic burdens observed in sham-immunized control groups[47,48].

CpG adjuvant typically promotes a Th1-type immune response[67]. Therefore, and as could be expected, the immunization procedure assessed here induced a predominant production of antigen-specific antibodies of the IgG2a isotype which is associated with a Th1-type immune response[57], although IgG1 antibodies were also raised by the i.n. immunization. As previously remarked, a balanced Th1/Th2 response might be more adequate in the course of neosporosis by conferring protection against the parasite, nonetheless avoiding fetal rejection[46]. Immunization with NcMP alone also induced the production of antigen-specific IgG. However, and in contrast with NcMP plus CpG immunization, this response was mainly characterized by the production of IgG1, indicative of a predominant Th2-type response. These results may indicate that NcMP antigens promote a Th2-type polarization of the immune response, associated with host susceptibility to neosporosis[68], that could be overcome by the usage of CpG adjuvant. A similar effect of CpG was previously observed in mice subcutaneously immunized with *N. caninum* lysates or soluble antigen preparations[28]. Our results also reinforce the adequateness of using CpG adjuvant to achieve immunoprotection against *N. caninum* infection as described by others using alternative immunization strategies[28,69].

Despite the fact that the serum IgG isotype profile might indicate that a Th1-type immune response was elicited in the NcMP/CpG group, no significant differences in the frequencies of splenic or MLN T cells producing IFN-γ, IL-10 and IL-4 could be detected among the studied groups. Since the majority of mice from the NcMP/CpG group presented no detectable parasitic colonization in the

absence of a significant increase in IFN-γ production the achieved protection could be, at least partially, independent of a strong Th1 response. Vaccine-induced protection in the absence of a Th1-type response was previously reported in mice infected by the protozoan *Leishmania amazonensis*[70].

Both immunized groups in this study presented parasite-specific IgA in the intestinal mucosa. Nevertheless, NcMP/CpG immunized mice presented higher IgA titres highlighting the mucosal adjuvant capacity of CpG also in the induction of IgA production. Still, although IgA is regarded as an hallmark of the induction of mucosal immunity[53,54], and even though primed cells in the nasal mucosa following i.n. immunization were shown to be capable of trafficking to the intestinal mucosa[71,72], in future works it would be worth confirming the presence of parasite-specific IgA producing cells in the intestinal lamina propria since transfer of IgA to the intestine from the serum through the bile has also been described in mice[73]. Nevertheless, in this study we have shown that parasite-specific IgA obtained from the intestinal lumen of immunized mice was capable of agglutinating *N. caninum* parasites. In this sense, and since previous studies have reported a host protective role of intestinal IgA in cats[74] and mice[75] infected with the closely related protozoan *Toxoplasma gondii*, it might be hypothesized that the lack of a noticeable Th1-type cytokine bias in the NcMP/CpG group could result from the impairment of host invasion across the intestinal mucosa due to parasite-agglutinating IgA that could also account for the higher number of mice with undetectable colonization levels. Supporting this hypothesis, the only animal in that group that did not present detectable levels of IgA in both analyzed mucosa was the one showing higher parasite DNA levels. In this scenario, although a cell mediated immune response, polarized to a Th1 phenotype, might be generated by the immunization its full activation upon infection in the immunized mice could have been prevented by lack of antigenic stimulation in the NcMP/CpG group. The lack of an exacerbated cellular immune response in the spleen and MLN of the NcMP/CpG group further support this hypothesis. In this sense, and as a strong production of IFN-γ in dams has previously been shown to compromise fetus viability[76,77], it would be valuable to assess if the immunizing protocol used herein could also be protective in a pregnant mice model of *N. caninum* infection.

In addition to the possible host-protective effects of IgA, we also show that IgG antibodies obtained from immunized mice can opsonize *N. caninum* tachyzoites and reduce parasite survival in murine macrophages challenged with this parasitic form. As *N. caninum* is an obligate intracellular pathogen, a cell-mediated rather than a humoral immune response could be expected to be host protective. However, these results indicate that antibodies specific for *N. caninum* might contribute for host protection against this parasite. These data are in agreement with previous reports demonstrating that antibodies specific for different membrane proteins of *N. caninum* are capable of interfering and even block the parasite entry in host cells[64-66]. However, such a protective role needs to be confirmed in further experiments, namely as the effect of IgG opsonization on leukocyte killing of tachyzoites of the closely related protozoan *T. gondii* remains controversial with previous studies showing either enhanced killing[78] or no effect in this regard[79].

Only a limited number of reports specifically studied the mucosal immune response to this parasite[47,48], and to the best of our knowledge no study had previously addressed the immune response in the intestinal mucosa and associated lymphoid tissue of mice immunized with *N. caninum* antigens. The widespread usage of i.p. inoculation in experimental studies on neosporosis likely contributed to the overlooking of the mucosal layer of immune defense against this parasitosis. By showing that intestinal lumen antibodies induced by mucosal immunization can agglutinate *N. caninum* tachyzoites we provide the first evidence indicating that stimulating antibody production in the gut by means of mucosal immunization may worth be attempted as a protective strategy against horizontally transmitted neosporosis.

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Chapter 5

Mucosal immunization confers long-term protection against intragastrically established *Neospora caninum* infection

Mucosal immunization confers long-term protection against intragastrically established *Neospora caninum* infection

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ABSTRACT

Neospora caninum is an obligate intracellular protozoan parasite responsible for severe economic losses in dairy and beef cattle farms. Although vaccination is widely regarded as the preferable strategy to control or prevent cattle neosporosis no commercial vaccine is currently available. We have previously shown that intranasal immunization with *N. caninum* membrane antigens and CpG adjuvant protected mice against intragastrically established neosporosis. Nevertheless, the long-term protective effect of this immunization strategy was not determined. Here, we show that the previously reported protective effect of this immunization procedure could still be observed 20 weeks upon immunization. This protection was accompanied by long-lasting elevated levels of parasite-specific serum IgG and intestinal IgA. In accordance with an observed IgG1/IgG2a ratio < 1, splenocytes from immunized mice responded to parasite-antigen recall by producing interferon-γ (IFN-γ). Nevertheless, one week after the infectious challenge no significant differences in IFN-γ production were observed between immunized mice and sham-immunized controls, regardless of the achieved protection. This work shows that the previously reported mucosal immunization strategy is effective in the long-term in protecting mice against *N. caninum* infection established through the gastrointestinal tract. Moreover, the results reported herein provide additional evidence suggesting that protection may primarily depend on antibody production.

INTRODUCTION

Neospora caninum is an apicomplexan protozoan closely related to *Toxoplasma gondii.* Initially recognized as the causative agent of neurodegenerative disease in dogs[1], *N. caninum* was further shown to infect a wide range of mammalian hosts among which cattle is the most economically relevant[2,3]. Recent estimates indicate that the annual worldwide economic losses in beef and dairy industries due to neosporosis, the disease caused by this parasite, surpass one billion dollars[4]. Transmission of *N. caninum* from infected cows to their fetuses is a major infection route[2,3] that accounts for the marked increase in the likelihood of abortion observed in infected dams[5-11]. However, ample evidence suggests that oral transmission to pregnant cows is responsible for 'storm-like' epidemic abortions[12]. Although economic analysis of different control strategies indicated that vaccination would be the most cost effective approach to manage this parasitic infection in cattle[13], no commercial vaccine is currently available in the market[14]. Nevertheless, natural infected dams can develop to some extent long-term protection against fetal loss in consecutive pregnancies[5,15,16]. In addition, inoculation with live and attenuated parasites have yield encouraging results regarding the prevention of vertical transmission and fetal loss in cattle indicating that a successful vaccine against this parasite can be achieved[17-20]. Nevertheless, despite their high effectiveness, vaccination with live attenuated strains have considerable drawbacks due to possible regression of the used strain to a more virulent status, as well shelf-life and logistical issues[20,21]. On the other hand, immunization with proteins involved in the parasite host cell invasion process are generally considered safe and have, for the most part, produced encouraging results in mice models of neosporosis, mainly leading to a reduction in parasitic burdens in infected animals[22-29]. Still, the success of these protocols in pregnant mice models of infection has shown some limitations in preventing vertical transmission or fetal loss[30,31].

Although the gastrointestinal tract constitutes the natural route of infection in horizontally transmitted neosporosis, and even though a recent characterization of the immune response in a mice model of intragastric infection showed that the parasite elicits both a mucosal and systemic immune response[32] (chapter 3),

mucosal immunization as a means to confer protection against this parasite has only been employed in a limited number of occasions despite encouraging results[33-35]. Recently, we have shown that intranasal (i.n.) immunization targeting *N. caninum* membrane proteins conferred protection against murine neosporosis established through the gastrointestinal tract[35] (chapter 4). Nevertheless, an ideal immunization protocol must prove effective in conferring long-lasting protection against the targeted pathogen. Therefore in this study, we evaluated whether the immune response and protective effect of our previously developed mucosal immunization strategy could be maintained in the long-term.

MATERIALS AND METHODS

Animals

Seven week-old female C57BL/6 mice were purchased to Charles River (Barcelona, Spain). Animals were kept at Instituto de Ciências Biomedicas Abel Salazar (ICBAS) animal facility under specific-pathogen free conditions throughout all the experimental procedures. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92). Authorization for the experiments was issued by the competent national board (document 0420/000/000/2010). Experiments were approved by the institutional board responsible for animal welfare (ORBEA) at ICBAS.

Parasites

N. caninum tachyzoites (Nc1 isolate) were kept by serial passages in VERO cells cultures, maintained in Minimal Essential Medium containing Earle's salts (Sigma, St. Louis, USA), supplemented with 10% fetal calf serum (Biowest, Nuaillé, France), L-Glutamine (2 mM), Penicillin (200 IU/ml) and Streptomycin (200 µg/ml) (all from Sigma), in a humidified atmosphere with 5% CO_2 at 37 ^{0}C. Tachyzoites were maintained until destruction of 80% of the host cell monolayer and were isolated as previously described[36]. Briefly, free parasites and adherent cells were recovered using a cell scraper and centrifuged at 1 500 × g for 15 min. The pellet was passed through a 25 G needle and then washed three times in PBS by centrifugation at 1 500 × g for 15 min. The resulting pellet was resuspended and passed through a PD-10 desalting column, containing Sephadex™ G-25M (GE Healthcare, Freiburg, Germany). Tachyzoites concentration was determined in a haemocytometer.

Preparation of whole tachyzoite lysates and cell-membrane extracts

N. caninum membrane proteins (NcMP) were extracted using a modification of a previously described method[37,38]. Briefly, free tachyzoites were resuspended in PBS containing 0.75% triton X-114 (Sigma), incubated 10 min on ice and centrifuged at 10 000 × g for 30 min at 4 ^{0}C and the supernatant was recovered and placed in a water bath at 30 ^{0}C during 3 min. The procedure was repeated and the supernatant was centrifuged at 1 000 × g for 3 min at room temperature. The aqueous phase was discarded and the NcMP were precipitated with the addition of absolute ethanol, vortexed vigorously during 15 sec and incubated 1 h on ice. The samples were centrifuged at 12 000 × g for 20 min at 4 ^{0}C and the resulting pellet was dried, resuspended in PBS and stored at -20 ^{0}C.

Whole *N. caninum* lysates were prepared by disruption of tachyzoites following sonication (20 cycles of 15 s at 100 W) with a Branson cell disrupter model W 185 D in an ice bath. The obtained *N. caninum* sonicates (NcS) were sequentially passed through 0.45 and 0.2 µm pore-size filters and stored at -20 ^{0}C. Quantification of NcMP or NcS was performed by using the Lowry protein assay. Sodium dodecyl sulphate polyacrylamide gel electrophoresis followed by silver nitrate staining was performed following each protein extraction in order to determine and confirm the protein migration profile.

Immunizations and tissue sample collections

Eight week-old female mice, were used with random distribution into 3 groups and immunized intranasally (i.n.) twice with three weeks interval under light isoflurane anesthesia with 20 µl of: PBS alone (PBS group, n=13) or PBS containing, 10 µg of CpG ODN 1826 VacciGrade (invivogen, San Diego, CA) (CpG group, n=13), or PBS containing 30 µg of NcMP plus 10 µg of CpG ODN 1826 VacciGrade (NcMP/CpG group, n=13). At weeks 1, 7 and 13 after the last immunization serum and vaginal lavages fluids (VLF) from all mice were collected for the detection of NcMP-specific IgG and IgA, respectively. At week 19, 3 animals per group were euthanized by cervical dislocation. Blood and intestinal lavage fluids (ILF) were collected for analysis of NcMP-specific IgG and IgA, respectively while the mesenteric lymph nodes (MLN) and spleen were aseptically removed for the

analysis of the immune response. All remaining mice were intragastrically (i.g.) challenged, 20 weeks after the last immunization, with 5×10^7 freshly isolated *N. caninum* tachyzoites as previously described[36]. One week after infection all the remaining mice were sacrificed by cervical dislocation and spleens were aseptically removed for the analysis of the immune response, while the brain was collected and stored at -20 ^{0}C for DNA extraction. At this day, serum and ILF from all mice were collected for the detection of *N. caninum* specific IgG and IgA, respectively.

Antibody detection

Serum IgG1 and IgG2a titres specific for NcMP were quantified by ELISA. Briefly, 96-well plates (Maxisorp, Nunc, Denmark), were coated overnight at 4 ^{0}C with NcMP diluted in PBS at a concentration of 5 µg/ml. All the wells were saturated with 2% bovine serum albumin (BSA) (Sigma) in TST buffer (150 mM NaCl, 10 mM EDTA and 0.05% Tween 20, pH=8) for 1 h. Serum samples were serial diluted in 1% BSA TST buffer and incubated for 1 h, followed by washing and addition of alkaline phospatase-coupled monoclonal goat anti-mouse IgG1 or IgG2a monoclonal antibodies (Southern Biotechnology Associates, Birmingham, USA) and incubation for 1 h. After washing, the specifically bound antibodies were detected by the development with the substrate solution of p-nitrophenyl phosphate (Sigma) and the reaction was stopped with the addition of 0.1 M EDTA, pH=8 solution. The absorbance was measured at 405 nm, subtracting for each well the value for the absorbance at 570 nm. The antibody titres were expressed as the $\log_{10}$ value of the reciprocal highest dilution with an absorbance higher than the value of the control (no serum added). Detection of IgA antibodies specific for NcMP were quantified by ELISA as previously described, using alkaline phospatase-coupled goat anti-mouse anti IgA mAb (Southern Biotech).

***In vitro* cell cultures and cytokine detection.**

To assess NcS-specific cytokine production spleens and MLN were aseptically removed from euthanized mice at the time-points previously described and homogenized in HBSS (Sigma). Spleen red blood cells were lysed and the spleen

and MLN cells were suspended in RPMI-1640 (Sigma), supplemented with 10% fetal calf serum (Biowest), HEPES (10 mM), Penicillin (200 IU/ml) and Streptomycin (200 µg/ml) (all from Sigma), β-mercaptoethanol (0.1 µM) (Merk) and plated (5×10^5/well) in triplicates per animal/organ in round bottom 96-well plates (Nunc), and stimulated with NcS (60 µg/ml) for 3 days at 37 ºC and 5% CO_2. Unstimulated conditions were set per animal/organ to assess the cells basal cytokine production level. The concentration of IFN-γ and IL-4 in cell culture supernatants were respectively quantified with the Mouse IFN-γ DuoSet® ELISA development system (R&D Systems, Minneapolis, MN, USA) and IL-4 ELISA Ready-Set-Go!® (eBioscience, San Diego, CA, USA) kits, both according to manufacturer's instructions.

DNA extraction

DNA from the brain of infected mice was extracted as previously described[39]. Briefly, brains were weighted and homogenized. Samples were incubated overnight at 55 ^{0}C in a solution containing 1% SDS and 1 mg/ml Proteinase K (sigma). DNA was extracted by the phenol (Sigma)-Chlorophorm (Merk) method followed by ammonium acetate/ethanol precipitation.

Real-time PCR analysis

The parasite burden in the brain of infected mice was assessed by quantitative real-time PCR (qPCR) analysis of the parasite DNA performed in a Corbett rotor gene 6000 system (Corbett life science, Sydney, Australia). Analysis was performed by using KAPA PROBE FAST 2xMasterMix, Universal (Grisp, Portugal), for the amplification of a 103 bp sequence of the Nc5 region of *N. caninum* genome using the primers NcA 5' GCTACCAACTCCCTCGGTT 3' and NcS 5' GTTGCTCTGCTGACGTGTCG 3' both at a final concentration of 0.2 µM, and the florescent probe FAM-CCCGTTCACACACTATAGTCACAAACAAAA-BBQ at a final concentration of 0.1 µM (all design and obtained from TIB-Molbiol, Berlin, Germany). The DNA samples were amplified using the following program: 95 ^{0}C for 3 min, 95 ^{0}C for 5 sec, 60 ^{0}C for 20 sec with fluorescence acquisition and the second and third steps were repeated 50 times. Length of the amplified DNA was

confirmed in a 3% agarose gel stained with ethidium bromide. In all runs parasite burden was determined by interpolation of a standard curve, ranging from 10 to 10^{-4} ng of DNA extracted from *N. caninum* tachyzoites included in each run and the data analyzed in the Rotor gene 6000 software v1.7 (Corbett life science).

Statistical analysis

Statistical analyses were performed using GraphPad prism, Version 5.0 (GraphPad Software, Inc., La Jolla, CA). In the scatter dot graphs the mean for each group was displayed as a horizontal bar. Column graphs are represented showing the mean plus one standard error of the mean (SEM). Statistical analysis between groups was performed by using one way ANOVA analysis of the variance with a Newman-Kleus post-hoc.

RESULTS

Intranasal immunization with NcMP induces long-lasting humoral immunity

In a previous work, we have shown that i.n. immunization with NcMP plus CpG adjuvant raised parasite-specific serum IgG and parasite-agglutinating intestinal IgA. Both showed effector function as the specific IgG facilitated *in vitro* parasite clearance by macrophages while the IgA antibodies strongly agglutinated *N. caninum* tachyzoites[35] (chapter 4). In order to determine whether the increased production of parasite-specific IgG and IgA antibodies induced by this immunization protocol could be maintained in the long term, mice were immunized by i.n. administration of 20 µl of PBS containing 30 µg NcMP and 10 µg CpG. Controls were sham-immunized with PBS alone or PBS plus CpG adjuvant. Prior to the infectious challenge, parasite-specific IgA production was assessed in VLF to monitor the effectiveness of immunization with minimal invasiveness, as previously reported[35] (chapter 4). As shown in Figure 5-1a, NcMP-specific IgA was detected in VLF samples collected from immunized mice at all assessed time-points. As expected no antigen-specific IgA was detected in similar samples obtained from sham-immunized controls. Nevertheless, and although elevated as compared to controls, NcMP-specific IgA titres in the VLF collected from immunized mice slowly decreased from week 1 to 13 upon the boost immunization. Still, analysis of IgA levels in ILF of non-infected mice collected 19 weeks after the boost immunization, showed these to be higher in the mucosa of immunized mice than in controls (Log_{10} IgA titre in NcMP/CpG group: 2.85±0.26 vs below detection limit in PBS and CpG sham-immunized groups; n=3 per group). As could be anticipated by this preliminary analysis, NcMP-specific IgA levels in ILF collected from immunized mice one week after i.g. infection revealed high NcMP-specific IgA levels, similar to those detected in the VLF one week after the boost immunization, while these were found below detection limit in both control mouse groups (Figure 5-1b).

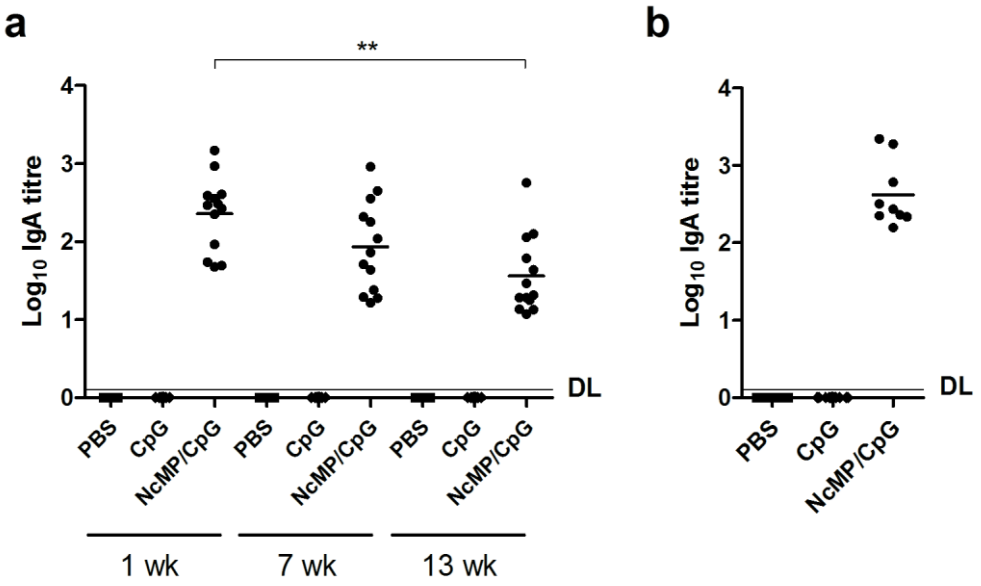

Figure 5-1 - Titres of *N. caninum* membrane proteins (NcMP)-specific IgA in vaginal and intestinal lavage fluids (VLF and ILF, respectively). IgA titres were determined by ELISA in (a) VLF collected one, seven and thirteen weeks upon boost immunization from mice immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant (CpG) or PBS alone and (b) in ILF collected from mice one week after i.g. challenge with 5×10^7 *N. caninum* tachyzoites. Data is presented as $\log_{10}$ of the antibody titres. Number of mice per VLF/ILF analyses were performed using: PBS n=13/9; CpG n=13/10; NcMP/CpG n=13/9. Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group; detection limit (DL) is indicated by a horizontal line; (* $p<0.05$).

In order to determine NcMP-specific IgG titres, serum samples were also collected from mice of all groups 1, 7 and 13 weeks following the boost immunization. As shown in Figure 5-2a, immunized mice serum NcMP-specific IgG antibodies were elevated in all time-points accessed without significant decrease in the mean titre of this immunoglobulin overtime. Parasite-specific IgG titres in sham-immunized control groups fell below detection limit in all assessed time-points. Analysis of the IgG isotype showed a mixed IgG1 and IgG2a production. Production of IgG1 and IgG2a are respectively associated with an underlying Th2- and Th1-type immune response[40]. As the IgG1/IgG2a ratio,

calculated with the mean titre values for each IgG isotype was lower than 1 in all analysis performed after the boost immunization, this indicates that the used immunization induced a Th1-type immune response bias. Serum parasite-specific IgG titres detected one week after the i.g. parasitic challenge were still below the detection limit in both sham-immunized groups while immunized mice presented a mixed IgG1 and IgG2a production with an IgG1/IgG2a ratio below 1 (Figure 5-2b). In summary, analysis of both IgG and IgA isotypes indicates that a long-lasting systemic and mucosal humoral response is elicited by i.n. immunization with NcMP and CpG.

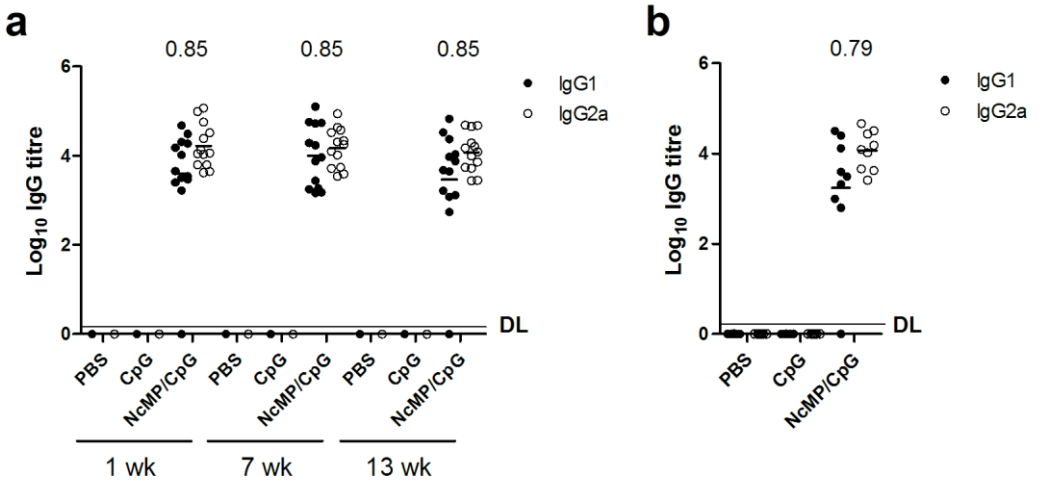

Figure 5-2 - Titres of *N. caninum* membrane proteins (NcMP)-specific IgG1 (closed circles) and IgG2a (open circles) in serum. IgG titres were determined by ELISA in serum collected (a) one, seven and thirteen weeks upon boost immunization and (b) one week after i.g. challenge with 5×10^7 *N. caninum* tachyzoites from mice immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant (CpG) or PBS alone. Data is presented as $\log_{10}$ of the antibody titres. Numbers above each group represent the IgG1/IgG2a ratio, calculated with the mean $\log_{10}$ titres for the correspondent IgG isotype. Number of mice per analysis in (a)/(b): PBS n=13/9; CpG n=13/10; NcMP/CpG n=13/9. Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group; detection limit (DL) is indicated by a horizontal line.

Long-lasting Th1-type immune memory was maintained upon immunization with NcMP

The previous IgG isotype analysis indicated that i.n. immunization with NcMP plus CpG adjuvant induced a parasite-specific biased Th1-type immune response. In order to confirm this indication and to analyze the memory response following antigen recall, spleen and MLN cells collected from immunized mice and controls 19 weeks after the boost immunization were stimulated *in vitro* with NcS in order to simulate the complex antigenic composition that these cells would encounter following host infection with *N. caninum*. As shown in Figure 5-3, a marked increase in IFN-γ levels was observed in the culture supernatants of NcS-stimulated splenocytes and MLN cells obtained from immunized mice, in comparison with similarly stimulated cells obtained from sham-immunized mouse groups. Regarding the levels of IL-4, and although IgG1 was found elevated in all time-points analyzed in the immunized group, these were found within control values, close to detection limits, in the NcS-stimulated splenocyte and MLN cultures in all assessed groups. The above results, altogether, indicate that parasite-specific immune memory persisted for at least 19 weeks following the boost immunization. Importantly, the memory response was found biased towards the production of host protective cytokine IFN-γ.

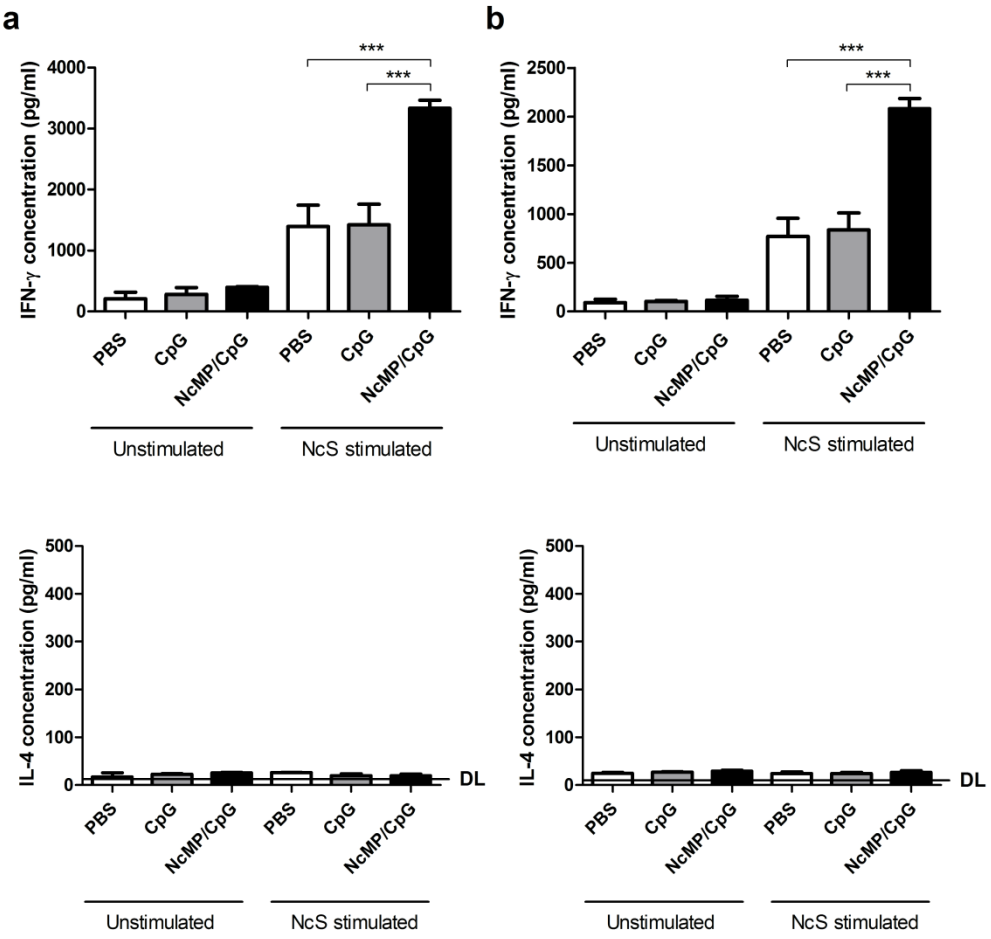

Figure 5-3 – IFN-γ and IL-4 concentration in the supernatants of (a) MLN or (b) splenocytes cell cultures unstimulated or stimulated for 3 days with *N. caninum* sonicates (NcS). Cells were isolated from the spleens and MLN of mice 19 weeks upon the last of two i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant (CpG) or PBS alone. Number of mice per analysis: PBS n=3; CpG n=3; NcMP/CpG n=3. Stimulated cells were placed in triplicates per mouse. Stimulated cells were placed in triplicates per mouse. Bars represent mean plus one SEM. Detection limit (DL) is indicated by a horizontal line (15 pg/mL) (*** $p<0.001$)

Long-term protection conferred by i.n. immunization against *N. caninum* infection

Having determined the persistence of immune memory, the long-term protective effect of i.n. immunization with NcMP plus CpG adjuvant was evaluated. In all groups, mice were infected i.g. with 5×10^7 *N. caninum* tachyzoites 20 weeks upon the boost immunization. One week after infection the parasitic burden was

assessed in the brain by parasite DNA-specific qPCR. As shown in Figure 5-4, a drastic reduction in the number of colonized animals could be observed in the NcMP/CpG group where all but mouse presented detectable parasite DNA. In contrast, high parasitic DNA levels were observed in the brain of the two sham-immunized control groups. This result shows that i.n. immunization with NcMP plus CpG confers long-lasting protection against *N. caninum* infection.

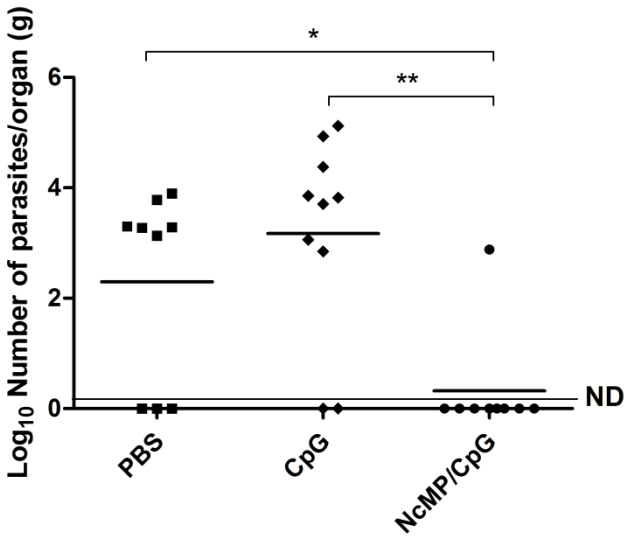

Figure 5-4 - Parasitic load assessed by qPCR one week upon i.g. challenge with 5×10^7 *N. caninum* tachyzoites in the brain of mice previously immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant (CpG) or PBS alone. Data is presented as $\log_{10}$ of the number of parasites by gram of organ. Number of mice per group: PBS n=9; CpG n=10; NcMP/CpG n=9. Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group; Not detected (ND) is indicated by a horizontal line; (* $p<0.05$; ** $p<0.01$)

Parasite-antigen recall induced a similar IFN-γ production by splenocytes of NcMP immunized mice and sham-immunized controls

One week upon the i.g. *N. caninum* challenge, the production of IFN-γ and IL-4 was evaluated in splenocyte cell cultures stimulated with NcS, by assessing the levels of these cytokines in the cell culture supernatants. As shown in Figure 5-5, cell culture NcS stimulation induced the production of IFN-γ in all assessed groups. However, no significant difference was found in the levels of IFN-γ between culture supernatants of splenocytes from immunized mice and PBS-

treated controls that were however significantly lower than the ones detected in the supernatants of cells collected from controls sham-immunized with CpG. The levels of IL-4 were found within control values in all groups. These results indicate that in the immunized mice the i.g. challenge did not induce a parasite-specific immune response, as assessed by IFN-γ production, more marked than that of controls sham-immunized with PBS. Results moreover show that CpG-priming, although not protective, predisposed the mice to respond with IFN-γ production when challenged with *N. caninum*.

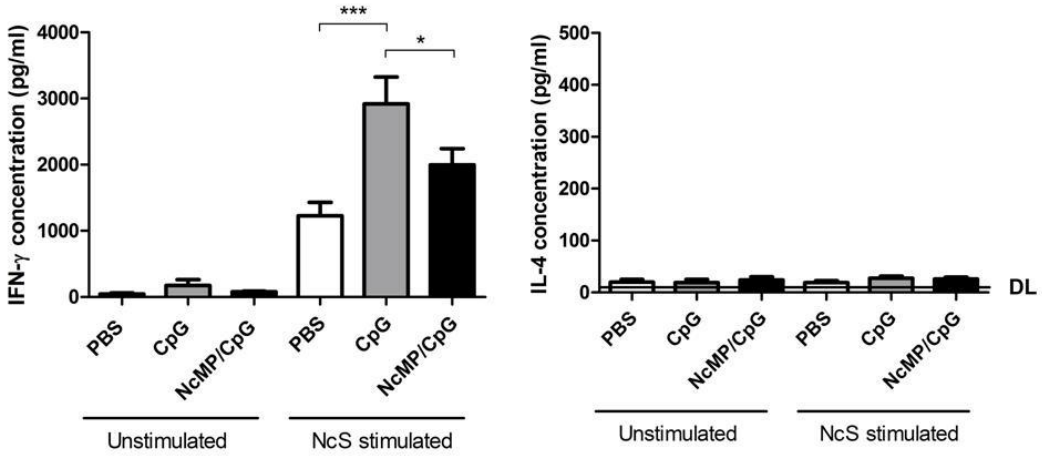

Figure 5-5 – IFN-γ and IL-4 concentration in the supernatants of splenocytes cell cultures unstimulated or stimulated for 3 days with *N. caninum* sonicates (NcS). Cells were isolated from the spleens of mice one week upon i.g. challenge with 5×10^7 *N. caninum* tachyzoites that have been previously immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant (CpG) or PBS alone. Number of mice per analysis: PBS n=9; CpG n=10; NcMP/CpG n=9. Stimulated cells were placed in triplicates per mouse. Bars represent mean plus one SEM. Detection limit (DL) is indicated by a horizontal line (15 pg/mL) (* $p<0.05$; *** $p<0.001$).

DISCUSSION

Cattle infection with *N. caninum* is a major cause of economic losses worldwide and although vaccination is regarded as the preferable option for the control of this parasite infection[13], the introduction in the market of an effective vaccine against this protozoan is still not foreseen in the near future[14]. Nevertheless, recent experimental immunization studies using either live attenuated strains or parasite subunits yielded promising results[14,41] suggesting that a viable vaccination strategy can be achieved. One factor that should be accounted in the development of an effective vaccine against neosporosis is that horizontal parasite transmission in cattle occurs via the gastrointestinal mucosa[2]. In fact, some studies have shown that horizontal transmission can occur more frequently than initially thought in cattle herds[42-44] and that this form of parasite transmission could be associated with the most severe cases of "abortion-storm" events[12]. Still, despite the recent evidence that following intragastric infection in mice *N. caninum* induces a mucosal and systemic immune response[32] (chapter 3) and that an ideal *N. caninum* vaccine should elicit both systemic and mucosal immunity[45], mucosal immunization in mice was only evaluated in a few studies[31,33,34] and has never been attempted in cattle in the context of neosporosis. In addition, the induction of a parasite-specific mucosal immune response as a means to achieve protection against this parasite has only been attempted in one study so far. In this regard, we have previously shown that i.n. immunization with NcMP as target antigen and CpG adjuvant could successfully protect mice challenged i.g. with *N. caninum*[35] (chapter 4). Here we further explored this immunization strategy by assessing the long-term elicited immune response and host protective effect. The induction of long-lasting memory is an essential point in the development of an effective immunization strategy that has never been addressed in previous studies on experimental vaccination against neosporosis. The obtained results showed that the used immunization strategy conferred long-lasting protection against i.g.-established neosporosis, still noticeable 20 weeks upon a single boost immunization. More importantly, and in agreement with our previous results[35] (chapter 4), parasitic DNA was detected only in the brain of a single immunized mouse. The achieved protection might

indicate that immunization prevented *N. caninum* penetrance across the gastrointestinal mucosa and/or elicited a parasite-specific immune response capable of clearing the infection. Still, further understanding of the exact host protective mechanisms elicited by this immunization protocol is necessary to clarify this point. Due to the unique immune regulation that occurs during pregnancy which could be a contributing factor for *N. caninum* high rate of vertical transmission[46,47], prevention of host infection could prove to be the best strategy to prevent *N. caninum* induced abortions in cattle.

We have previously determined that the humoral immune response elicited by the used immunization involved production of parasite-agglutinating IgA in the vaginal and intestinal mucosa, and parasite-opsonizing serum IgG which facilitated *in vitro* parasite clearance by bone-marrow-derived macrophages[35] (chapter 4). Therefore, we assessed here whether parasite-specific Ig of both isotypes could still be produced in the long-term upon immunization. IgA produced in mucosal surfaces is regarded as a hallmark of mucosal immunity[48-50] namely due to its immune exclusion function through pathogen agglutination[48,50-52]. Therefore, the parasite-specific intestinal IgA elicited by immunization might be a key contributor to the observed protection by promoting parasite agglutination and prevention of intestinal epithelial barrier penetration. Still, in this study we found that the levels of parasite-specific IgA in the vaginal mucosa steadily decreased over-time though its mean titre was still well above control levels 13 weeks following the boost immunization.

Previous studies addressing the kinetics of IgA production in the intestinal mucosa indicated that in the absence of an antigen-specific stimulus the production of antigen-specific IgA decreases over-time due to a dynamic modulation of the population of IgA producing plasma cells in the mucosa lamina propria where newly arrived IgA producing cells displace previously resident cells[53]. In accordance, a previous work showed that in mice i.n. immunized with *T. gondii* antigen SAG1 plus cholera toxin adjuvant antigen-specific IgA production in the gut gradually decreased in the 150 days following the boost immunization. This decrease could be partially reversed by an additional boost immunization[54]. Similarly, in mice inoculated i.g. with rotavirus, virus-specific IgA could be detected in the intestine 10 months after priming although at 3-fold lower levels than at 42

days after infection[55]. Here, parasite-specific IgA levels detected in ILF of immunized mice, either 19 weeks after the boost immunization in uninfected mice or one week after infection were similar to those detected 4 weeks after the boost immunization, as previously reported[35] (chapter 4). This indicates that parasite-specific IgA production in the intestinal mucosa induced by the NcMP plus CpG prime-boost immunization was sustained for at least 20 weeks. In agreement, mice i.n. immunized with *Yersinia pestis* peptides plus CpG adjuvant displayed sustained intestinal IgA production, detected for at least 120 days upon immunization[56]. This report in conjunction with the results shown here could indicate that CpG adjuvant might induce long-lasting IgA production in the intestinal mucosa following i.n. immunization. Furthermore, following oral immunization with cholera toxin IgA-producing memory cells could still be observed 1 year later in the MLN, although toxin-specific IgA production was found to decrease over time in the intestinal mucosa[57].

In this study we also observed that in the immunized mice, parasite-specific IgG titres were significantly elevated in the long term showing no significant decrease overtime. Several studies have indicated that IgG raised against *N. caninum* surface and apical vesicle complex proteins could prevent parasite invasion of host cells[58-62]. Moreover, and in line with these results, we have previously reported that NcMP-specific serum IgG elicited by the immunization procedure used here also displayed *in vitro* effector function[35] (chapter 4). Therefore, it can be hypothesized that the IgG raised by the immunization might also contribute to host protection. Still, and to the best of our knowledge, protection conferred by *N. caninum*-specific IgG has never been shown *in vivo* in either mice or cattle, making it an interesting point to be explored in future studies. In addition, and in accordance with our previous results[35] (chapter 4) isotype profile of the produced IgG showed a preponderant production of IgG2a over IgG1, indicative of a Th1-type response bias[40]. Nevertheless, in this study it was found that this higher IgG2a production was sustained in all subsequent analysis performed, indicating the induction of a long-lasting biased Th1-type immune response in the NcMP immunized mice. In agreement, IFN-γ and IL-4 produced by MLN and spleen cells of the immunized mice upon *in vitro* parasite-specific antigen recall 19 weeks after the boost immunization, confirmed the Th1-type bias

by showing a clear increase in the production of IFN-γ and negligible IL-4 production, as compared to sham-immunized groups. These results also confirm the well-known Th1-type immunomodulatory capacity of the CpG adjuvant when used as a mucosal adjuvant[63-65]. In addition, and due to the known host protective role of IFN-γ in *N. caninum* infection[66-68], recently shown to be the main mediator of host protection conferred by $CD8^+$ T cells[69], the induction of a memory IFN-γ producing response could be a contributing factor to the observed protection. Nevertheless, the same analysis performed using splenocytes obtained one week after the i.g. infection showed that IFN-γ production in sham-immunized mice reached levels similar to those of immunized mice. A similar observation was made in mice infected 3 weeks upon the boost immunization that was previously suggested to be the result of IgA-mediated prevention of parasite penetration in the host across the gastrointestinal tract and consequent deprivation of antigen stimulation in the immunized mice[35] (chapter 4). In fact, while the mean IFN-γ levels in both sham-immunized groups increase following mice infection when compared with pre-infection levels, the mean IFN-γ concentration detected one week after infection in immunized mice was similar to that previously observed in similarly stimulated splenocytes prior to infection. This would be in agreement with the observation reported here that all but one of the immunized mice presented no detectable parasitic DNA in the brain.

Altogether, in this study we have used a mice model as proof of the concept to assess the long-term protective effect of our i.n. immunization protocol using NcMP and CpG adjuvant. We have successfully shown that long-lasting protection accompanied by a mucosal and systemic humoral and cell-mediated parasite-specific immune response could be induced in i.n. immunized mice. This result further strengthens the robustness of our immunization strategy in the mice model of neosporosis which makes it a prime candidate for a future evaluation of its immunogenicity and protective effect in cattle

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Chapter 6

Mucosal immunization confers protection against infection with the intracellular apicomplexa *Neospora caninum* in a highly susceptible IL-12p40$^{-/-}$-deficient mice model

Mucosal immunization confers protection against infection with the intracellular apicomplexa *Neospora caninum* in a highly susceptible IL-12p40$^{-/-}$-deficient mice model

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ABSTRACT

Neospora caninum is an obligate intracellular apicomplexa protozoan that is associated with high economic losses due to abortions induced by neosporosis in cattle. Despite the worldwide distribution of this parasitic infection in cattle and that vaccination is regarded as the preferable option for its control, no commercial vaccine is currently available. In this regard, we have recently demonstrated the protective effect of an intranasal immunization protocol, using *N. caninum* membrane proteins, in a murine model of intragastrically established neosporosis. Regardless of the intracellular nature of this pathogen, the obtained results hinted that the achieved protection could be mediate by a parasite-specific mucosal and systemic humoral immune response in the absence of a marked Th1-type cellular immunity. As such, and in order to confirm this hypothesis, we determined if protection could be achieved in immunized mice lacking IL-12/IL-23 p40 chain (IL-12p40$^{-/-}$) expression. Immunized mice presented increased protection following intragastric or intraperitoneal infection with *N. caninum* tachyzoites. Protection was accompanied by a mucosal and systemic parasite-specific immune response. Although high levels of interferon-γ were detected following infection, no significant differences between immunized and sham-immunized controls were observed. These results indicate that our immunization strategy is capable of conferring protection in a highly susceptible mouse model with compromised cell-mediated immunity.

INTRODUCTION

Neospora caninum is an obligate intracellular apicomplexa protozoan initially described in dogs presenting several neuromuscular disorders[1]. In the following decades, dogs[2] and other canids[3-5] have been described as definitive hosts. However, the parasite infects a wide range of intermediate hosts from which cattle is recognized as the most relevant[6-8]. Cattle infection with *N. caninum* is associated with high economic losses[9] due to an increased abortion rate observed in infected animals[10-16] as a consequence of the parasite high rate of vertical transmission[16-22]. Even though infected herds have been described worldwide[6,7] and economic losses are estimated to be as high as 2 billion dollars/annum[9], no effective or economically viable control option to manage cattle neosporosis is currently available[23,24]. Nevertheless, economic models indicate that the development of an effective vaccine that could either prevent the primary infection or vertical transmission of this parasite could constitute a viable cost effective strategy to control neosporosis[23,24]. Since other successful vaccines against closely related apicomplexa protozoans are currently available[25,26], there is a consensus on the feasibility to develop an effective vaccine against *N. caninum*.

As previously mentioned, *N. caninum* is an intracellular protozoan and as such it can be expected that a Th1-type cell-mediated immunity should be essential for parasite control. In this regard, several studies have shown that defects in the IL-12/IFN-γ axis rendered mice lethally susceptible to the parasite infection[27-32]. Nevertheless, B cells were previously shown to be essential for host resistance against *N. caninum* infection, indicating that antibodies should have a role to play in host protection[33]. In fact, several studies have confirmed that antibodies against proteins that mediate parasite attachment and invasion of host cells are capable preventing *N. caninum* infection of host cells *in vitro*[34-40].

Although *N. caninum* primary infection in cattle is established through the gastrointestinal tract, very few studies have previously characterize or considered the host immune response in the gastrointestinal mucosa following parasite infection. Nevertheless, it is known that intragastric (i.g.) infection with *N. caninum* tachyzoites elicits the production of parasite-specific intestinal IgA and serum

IgG[41] in addition to an early increase production of IFN-γ in intestinal epithelial lymphocytes and in the draining mesenteric lymph nodes (MLN)[42] (chapter 3). Still, in spite of these studies, and although the induction of a parasite-specific mucosal immune response could prove a prime strategy to achieve host protection against *N. caninum* infection, very few studies have attempted mucosal immunization as a means to confer protection against this parasite[43-45] and in those studies, the possible protective effect of the elicited mucosal immune response was overlooked. In this regard, the protective effect of intranasal (i.n.) immunization using *N. caninum* membrane proteins (NcMP) and CpG as adjuvant in conferring protection against i.g. established neosporosis in mice was recently demonstrated[46] (Chapter 4 and 5). The observed protection was largely characterized by undetectable parasite colonization in immunized mice which could indicate that primary infection was averted or efficiently cleared. In addition, protection appeared to be mediated by parasite-agglutinating intestinal IgA and parasite-opsonizing serum IgG. Supporting this hypothesis, immunized mice did not present a significant increase in IFN-γ production compared to sham-immunized controls, indicating that the achieved protection was to some extent independent of a robust Th1-type immune response[46] (Chapter 4). As such, in this study, and in order to confirm our hypothesis and further characterize the protective mechanism elicited by our immunization protocol we analyzed its protective effect in IL-12p40$^{-/-}$ mice which are highly susceptible to *N. caninum* infection due to their compromised Th1-type cell-mediated immunity[32].

MATERIALS AND METHODS

Animals

Seven week-old female C57BL/6 IL-12p40$^{-/-}$ mice were purchased from Jackson laboratories (Bar Harbor, Maine, USA). Animals were kept under specific pathogen-free conditions and bred at Instituto de Ciências Biomédicas Abel Salazar (ICBAS) animal facility. Animals were maintained in the same installations throughout all the experimental procedures. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92). Authorization for the experiments was issued by the animal welfare section of the competent national board, Direcção Geral de Veterinária (0420/000/000/2008, 0420/000/000/2010). Experiments were approved by the institutional board responsible for animal welfare (ORBEA) at ICBAS.

Parasites

N. caninum tachyzoites (Nc1 isolate) were kept by serial passages in VERO cells cultures, maintained in Minimal Essential Medium containing Earle's salts (Sigma, St. Louis, USA), supplemented with 10% fetal calf serum (Biowest, Nuaillé, France), L-Glutamine (2 mM), Penicillin (200 IU/ml) and Streptomycin (200 µg/ml) (all from Sigma), in a humidified atmosphere with 5% CO_2 at 37 ^{0}C. Tachyzoites were maintained until destruction of 80% of the host cell monolayer and were isolated as previously described[41]. Briefly, free parasites and adherent cells were recovered using a cell scraper and centrifuged at 1 500 × g for 15 min. The pellet was passed through a 25 G needle and then washed three times in PBS by centrifugation at 1 500 × g for 15 min. The resulting pellet was resuspended and passed through a PD-10 desalting column, containing Sephadex™ G-25M (GE Healthcare, Freiburg, Germany). Tachyzoites concentration was determined in a haemocytometer.

Preparation of whole tachyzoite lysates and cell-membrane extracts

N. caninum membrane proteins (NcMP) were extracted using a modification of a previously described method[47,48]. Briefly, free tachyzoites were resuspended in PBS containing 0.75% triton X-114 (Sigma), incubated 10 min on ice and centrifuged at 10 000 × g for 30 min at 4 ^{0}C and the supernatant was recovered and placed in a water bath at 30 ^{0}C during 3 min. The procedure was repeated and the supernatant was centrifuged at 1 000 × g for 3 min at room temperature. The aqueous phase was discarded and the NcMP were precipitated with the addition of absolute ethanol, vortexed vigorously during 15 sec and incubated 1 h on ice. The samples were centrifuged at 12 000 × g for 20 min at 4 ^{0}C and the resulting pellet was dried, resuspended in PBS and stored at -20 ^{0}C.

Whole *N. caninum* lysates were prepared by disruption of tachyzoites following sonication (20 cycles of 15 s at 100 W) with a Branson cell disrupter model W 185 D in an ice bath. The obtained *N. caninum* sonicates (NcS) were sequentially passed through 0.45 and 0.2 µm pore-size filters and stored at -20 ^{0}C. Quantification of NcMP or NcS was performed by using the Lowry protein assay. Sodium dodecyl sulphate polyacrylamide gel electrophoresis followed by silver nitrate staining was performed following each protein extraction in order to determine and confirm the protein migration profile.

Immunizations and tissue sample collection

Eight week-old female mice, were used in 2 independent experiments with random distribution of mice into 2 groups and immunized i.n. twice with three weeks interval under light isoflurane anesthesia with 20 µl of PBS containing, 10 µg of CpG ODN 1826 VacciGrade (Invivogen, San Diego, CA) (CpG group, n=16), or PBS containing 30 µg of NcMP plus 10 µg of CpG ODN 1826 VacciGrade (NcMP/CpG group, n=18). Vaginal lavages fluids (VLF) and serum samples were collected 1 week after the boost immunization for the detection of NcMP-specific IgA and IgG, respectively. All mice were i.g. challenged, 3 weeks after the last immunization, with 5 × 10^7 freshly isolated *N. caninum* tachyzoites as previously described[41]. One week after infection all mice were sacrificed by cervical dislocation and spleens were aseptically removed for the analysis of the immune

response, while the brain was collected and stored at -20 ^{0}C for DNA extraction. Additionally, serum and intestinal lavage fluids (ILF) were collected from all mice for the detection of NcMP-specific IgG and IgA, respectively.

Eight week-old female mice were also similarly immunized in 2 independent experiments (CpG, n=14 and NcMP/CpG, n=14) and samples were collected at the same time-points as described above. These mice were however infected intraperitoneally (i.p.) with 1×10^4 freshly isolated *N. caninum* tachyzoites.

Antibody detection

Serum titres of NcMP-specific IgG1 and IgG2a were quantified by ELISA. Briefly, 96-well plates (Maxisorp, Nunc, Denmark) were coated overnight at 4 ^{0}C with NcMP diluted in PBS at a concentration of 5 µg/ml. All the wells were saturated with 2% bovine serum albumin (BSA) (Sigma) in TST buffer (150 mM NaCl, 10 mM EDTA and 0.05% Tween 20, pH=8) for 1 h. Serum samples were serially diluted in 1% BSA TST buffer and incubated for 1 h, followed by washing and addition of alkaline phospatase-coupled monoclonal goat anti-mouse IgG1 or IgG2a monoclonal antibodies (Southern Biotechnology Associates, Birmingham, USA) and incubation for 1 h. After washing, the specifically bound antibodies were detected by the development with the substrate solution of p-nitrophenyl phosphate (Sigma) and the reaction was stopped with the addition of 0.1 M EDTA, pH=8 solution. The absorbance was measured at 405 nm, subtracting for each well the value for the absorbance at 570 nm. The antibody titres were expressed as the $\log_{10}$ value of the reciprocal highest dilution with an absorbance higher than the value of the control (no serum added). Detection of IgA antibodies specific for NcMP were quantified by ELISA as previously described, using alkaline phospatase-coupled goat anti-mouse anti-IgA (Southern Biotech).

Purification of serum IgG antibodies and passive immunization

Mouse serum samples collected at the day of euthanasia were used for IgG purification. Two pools of sera were prepared using samples collected from previously immunized mice of the NcMP/CpG and CpG groups, and respectively named IgG-NcMP and IgG-CpG. For each of the prepared pools, IgG antibody

purification was carried out using a HiTrap Protein G HP purification column (GE healthcare), according to manufacturer's instructions. Recovered antibodies were buffer exchanged against sterile PBS, normalized to a final concentration of 1.5 mg/ml, using the Lowry protein assay and stored at -20 ^{0}C. The NcMP-specific antibody titre of the IgG-NcMP and IgG-CpG preparations were of 1.559 × 10^{9} and below the detection limit, respectively, as determined by ELISA. Passive immunization was performed in eight week-old female mice randomly distribution into 2 groups per experiment. Purified IgG-CpG (IgG-CpG group, n=5) and IgG-NcMP (IgG-NcMP group, n=5) fractions were transferred by intravenous (i.v.) injection (200 µg of total IgG per mice). Twelve hours following IgG transfer all mice were infected i.p. with 1 × 10^{4} freshly isolated *N. caninum* tachyzoites. Mice were sacrificed one week after infection by cervical dislocation and the brains were collected and stored at -20 ^{0}C for DNA extraction.

In vitro cell cultures and cytokine detection

To assess NcS-specific cytokine production, spleens were aseptically recovered from euthanized mice at specific time-points and homogenized. Spleen red blood cells were lysed and recovered splenocytes were suspended in RPMI-1640 (Sigma), supplemented with 10% fetal calf serum (Biowest), HEPES (10 mM), Penicillin (200 IU/ml) and Streptomycin (200 µg/ml) (all from Sigma) and β-mercaptoethanol (0.1 µM) (Merk, Darmstadt, Germany) (complete RPMI), plated (5 × 10^{5}/well) in triplicates per animal in round bottom 96-well plates (Nunc) and stimulated with NcS (60 µg/ml) for 3 days at 37 ºC and 5% CO_2. Unstimulated conditions were set per animal to assess the cells basal cytokine production level. The concentration of IFN-γ and IL-4 in cell culture supernatants were respectively quantified with the Mouse IFN-γ and IL-4 ELISA Ready-Set-Go!® (eBioscience, San Diego, CA) kits, both according to manufacturer's instructions.

Intracytoplasmic staining

For intracellular cytokine detection by flow cytometry, spleen cells previously obtained in complete RPMI were plated in round bottom 96-well plates (1 × 10^{6} cells/well) (Nunc). Cells were incubated in a humidified atmosphere with 5% CO_2

at 37 ^{0}C for 5 h under stimulation with 20 ng/ml phorbol myristate acetate (Sigma), 200 ng/ml ionomycin (Merk) and 10 ng/ml Brefeldin A (Sigma). Following stimulus, cells were recovered and unspecific antibody binding was prevented by the pre-incubation with anti-FcγR mAb followed by incubation with either anti-CD4 Peridinin-chlorophyll proteins-cychrome 5.5-conjugate (clone RM4-5) or anti-CD8 Phycoeritrin-conjugate (clone 53-6.7) mAb (both from BDbiosciences, San Diego, CA). Following extracellular staining the cells were washed, fixed in 2% formaldehyde (Merck), washed again and permeabilized with 0.05% saponin (Sigma) PBS solution and intracytoplasmic staining was carried out with anti-IFN-γ fluorescein isothiocyanate-conjugate (clone XMG1.2, BDbiosciences) after pre-incubation of the cells with anti-FcγR mAb. Antibody-labeled cells were analyzed in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter, Miami, FL). At least 150,000 events were acquired per sample. The collected data files were converted using FACS convert, v1.0 (Becton Dickinson, San Diego, CA) and analyzed using Cell Quest software, v3.2.1f1 (Becton Dickinson).

DNA extraction

DNA from the brain of infected mice was extracted as previously described[49]. Briefly, brains were weighted and homogenized. Samples were incubated overnight at 55 ^{0}C in a solution containing 1% SDS and 1 mg/ml Proteinase K (sigma). DNA was extracted by the phenol (Sigma)-Chlorophorm (Merk) method followed by ammonium acetate/ethanol precipitation.

Real-time PCR analysis

The parasite burden in the brain of infected mice was assessed by quantitative real-time PCR (qPCR) analysis of the parasite DNA performed in a Corbett rotor gene 6000 system (Corbett life science, Sydney, Australia). Analysis was performed by using KAPA PROBE FAST 2xMasterMix, Universal (Grisp, Portugal), for the amplification of a 103 bp sequence of the Nc5 region of *N. caninum* genome using the primers NcA 5’ GCTACCAACTCCCTCGGTT 3’ and NcS 5’ GTTGCTCTGCTGACGTGTCG 3’ both at a final concentration of 0.2 μM,

and the florescent probe FAM-CCCGTTCACACACTATAGTCACAAACAAAA-BBQ at a final concentration of 0.1 µM (all design and obtained from TIB-Molbiol, Berlin, Germany). The DNA samples were amplified using the following program: 95 ^{0}C for 3 min, 95 ^{0}C for 5 sec, 60 ^{0}C for 20 sec with fluorescence acquisition while the second and third steps were repeated 50 times. Length of the amplified DNA was confirmed in a 3% agarose gel stained with ethidium bromide. In all runs parasite burden was determined by interpolation of a standard curve, ranging from 10 to 10^{-4} ng of DNA extracted from *N. caninum* tachyzoites included in each run and the data analyzed in the Rotor gene 6000 software v1.7 (Corbett life science).

Statistical analysis

Statistical analyses were performed using GraphPad prism, Version 5.0 (GraphPad Software, Inc., La Jolla, CA). In the scatter dot graphs the mean for each group was displayed as a horizontal bar. Column graphs are represented showing the mean plus one standard error of the mean (SEM). Statistical analysis between groups was performed by using student's *t*-test.

RESULTS

Production of parasite-specific mucosal IgA and serum IgG in immunized p40$^{-/-}$ mice

In previous studies[46] (Chapter 4 and 5), i.n. immunization of C57Bl/6 WT mice with NcMP and CpG resulted in the production of parasite-specific intestinal IgA and serum IgG, respectively capable of agglutinating and opsonizing *N. caninum*. Considering the possible host protective effect of these immunoglobulins, we assessed if these could also be detected in the mucosa and serum of similarly immunized p40$^{-/-}$ mice. As shown in Figure 6-1a and 6-2a, one week after the boost immunization, and as expected, no parasite-specific IgA or IgG in VLF and serum samples respectively, were detected in sham-immunized mice receiving CpG alone. In comparison, immunized mice presented parasite-specific VLF IgA and serum IgG. IgG isotype analysis in the immunized group revealed a mixed IgG1 and IgG2a production. Since production of IgG1 and IgG2a can respectively be associated with an underlying Th2- and Th1-type immune response[50], the analysis of these immunoglobulin isotypes can be an indicator of the type of elicited cellular immune response. The IgG1/IgG2a ratio in immunized mice, calculated with the mean titres values for each IgG isotype, was below 1 indicating that a small bias towards a Th1-type immune response was induced in p40$^{-/-}$ mice upon the i.n. immunization.

One week after either i.g. (Figure 6-1b and 6-2b) or i.p. (Figure 6-1c and 6-2c) infection with *N. caninum* tachyzoites, and as indicated by the VLF analysis, parasite-specific IgA could be detected in ILF samples of all immunized mice. In accordance, parasite-specific IgG titres were also detected in the serum of NcMP/CpG mice, and in this instance an IgG1/IgG2a < 1 could still be observed. In both cases no parasite-specific IgA or IgG was detected in sham-immunized controls. Altogether, these results show that i.n. immunization with NcMP and CpG is capable of inducing the production of parasite-specific mucosal IgA and serum IgG in a p40$^{-/-}$ mice with a small bias towards a Th1-type immune response.

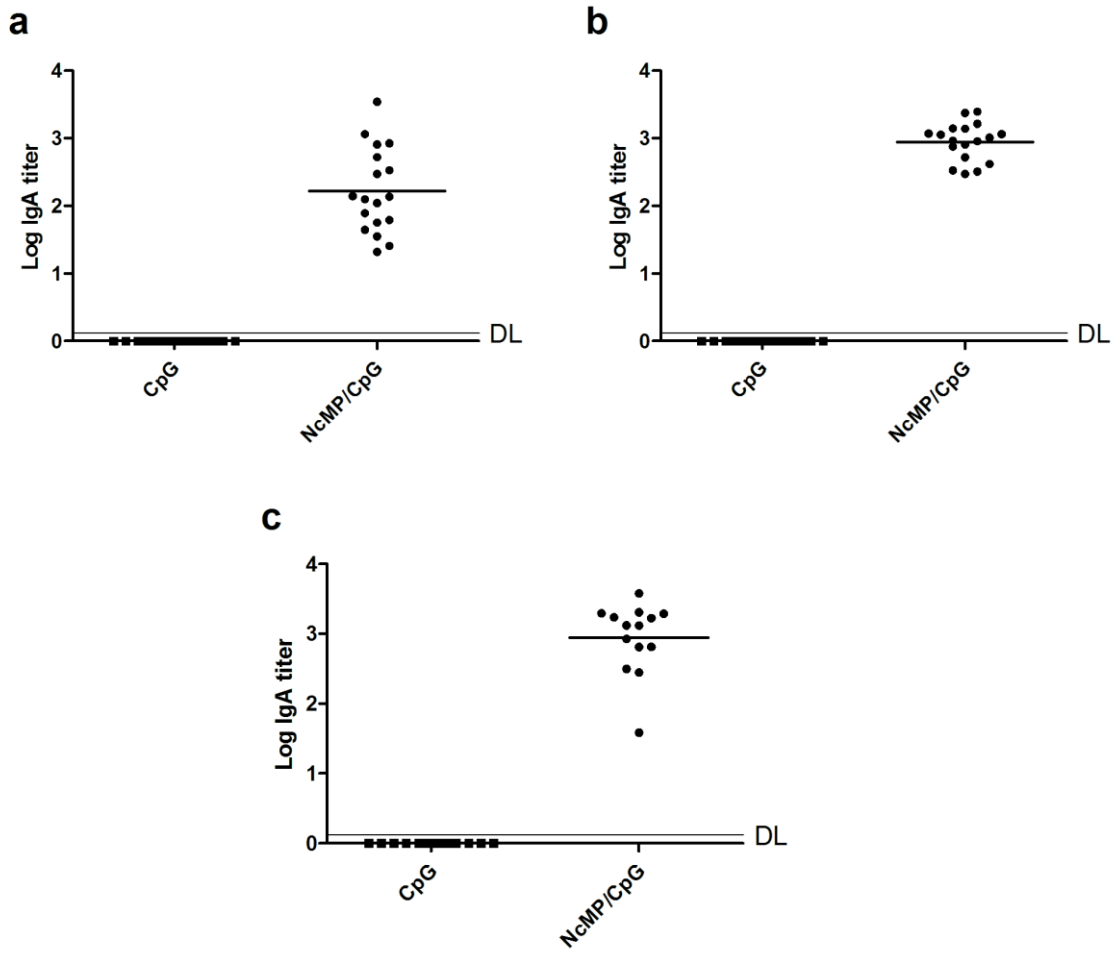

Figure 6-1 - Titres of *N. caninum* membrane proteins (NcMP)-specific IgA in vaginal and intestinal lavage fluids (VLF and ILF, respectively). IgA titres were determined by ELISA in (a) VLF collected one week upon boost immunization from mice immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG) or (b) ILF collected from mice one week after i.g. challenge with 5×10^7 *N. caninum* tachyzoites or (c) ILF collected from mice one week after i.p. challenge with 1×10^4 *N. caninum* tachyzoites. Data is presented as $\log_{10}$ of the antibody titres, as indicated. Results correspond to pooled data of two independent experiments with a total number of mice per group of: CpG n=16; NcMP/CpG n=18 for i.g. infected mice and CpG n=14; NcMP/CpG n=14 for i.g. infected mice. Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group; detection limit (DL) is indicated by a horizontal line.

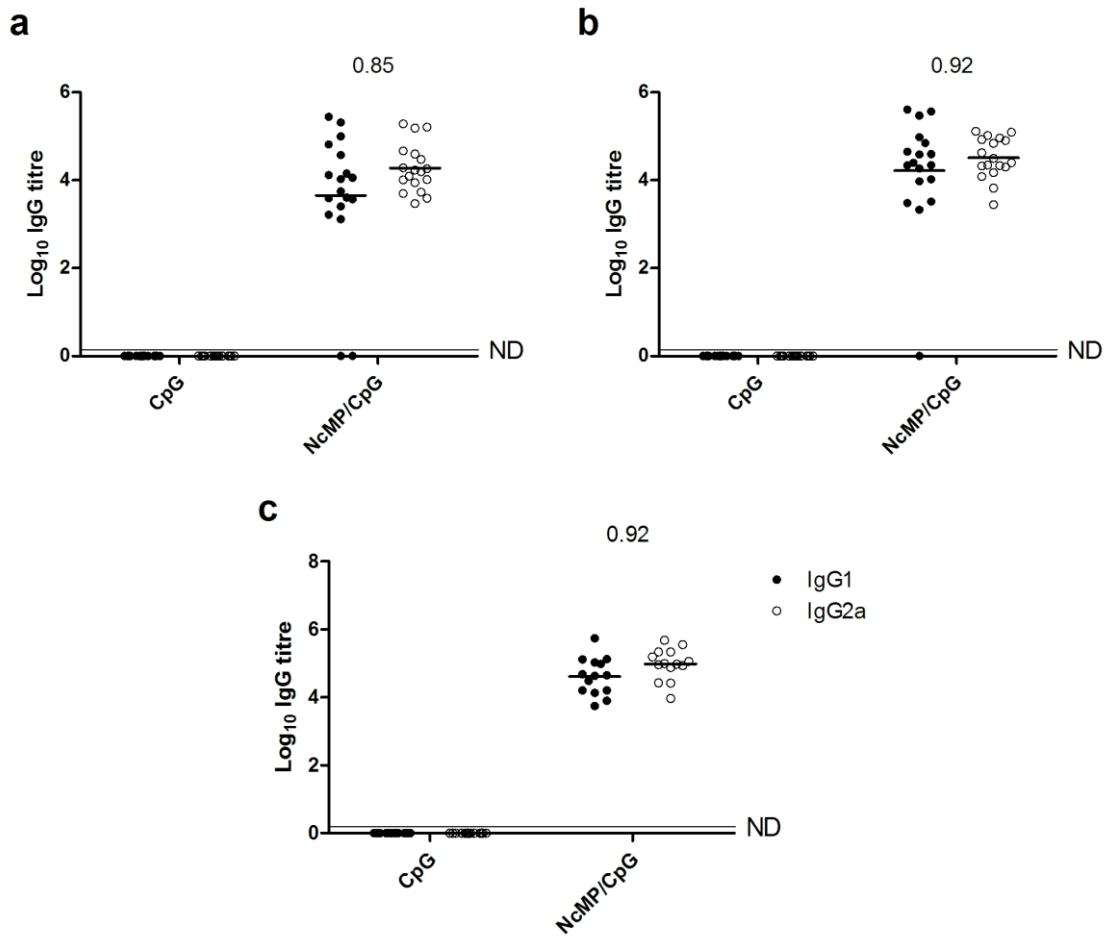

Figure 6-2 - Titres of *N. caninum* membrane proteins (NcMP)-specific IgG1 (closed circles) and IgG2a (open circles) in serum. IgG titres were determined by ELISA in serum collected one week upon boost immunization (a) from mice immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG) or (b) from mice serum one week after i.g. challenge with 5×10^7 *N. caninum* tachyzoites or (c) from mice serum one week i.p. challenge with 1×10^4 *N. caninum* tachyzoites. Data is presented as $\log_{10}$ of the antibody titres, as indicated. Numbers above each group represent the IgG1/IgG2a ratio, calculated with the mean $\log_{10}$ titres for the correspondent IgG isotype. Results correspond to pooled data of two independent experiments with a total number of mice per group of: CpG n=16; NcMP/CpG n=18 for i.g. infected mice and CpG n=14; NcMP/CpG n=14 for i.g. infected mice. Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group; detection limit (DL) is indicated by a horizontal line.

Prevention of parasite colonization in immunized p40$^{-/-}$ mice following i.g. infection

We have previously demonstrated that the i.n. immunization strategy used here was capable of conferring protection to WT mice against i.g. established neosporosis in the absence of increase IFN-γ production[46] (chapter 4). As p40$^{-/-}$ mice are lethally susceptible to *N. caninum* infection[32], we assessed if similar i.n. immunization with NcMP and CpG in p40$^{-/-}$ mice could also protect this host against i.g. established neosporosis. Sham and immunized mice groups were i.g. infected with 5 × 10^7 *N. caninum* tachyzoites 3 weeks after the boost immunization. One week after the parasitic challenge we assessed the brain parasitic burden by qPCR. As shown in Figure 6-3, a clear reduction in the number of animals colonized in this organ was observed in the NcMP/CpG group with 9/18 mice negative for the presence of *N. caninum* DNA. In comparison, only 2/16 mice in the CpG group were negative for the presence of *N. caninum* DNA. Nevertheless, similar parasitic burdens were detected in the colonized mice of sham- and immunized groups. Overall this result shows that protection against i.g. established neosporosis could be achieved following i.n. immunization of p40$^{-/-}$ mice, characterized by an absence of detectable colonization levels in protected mice.

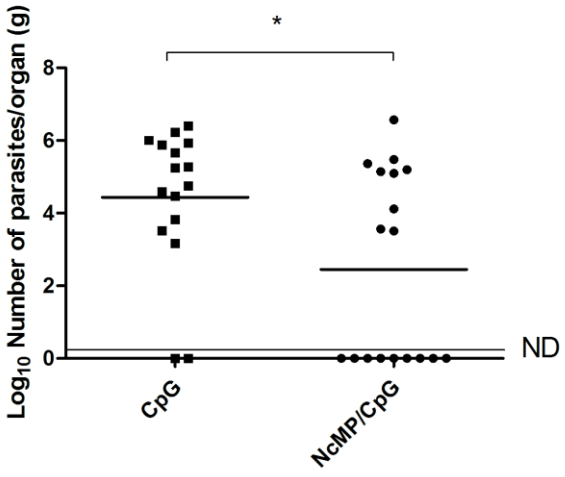

Figure 6-3 - Parasitic load assessed by qPCR one week upon i.g. challenge with 5 × 10^7 *N. caninum* tachyzoites in the brain of mice previously immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG). Data is presented as $\log_{10}$ of the number of parasites by gram of organ. Results are from pooled data of two independent experiments. Total number of mice per group: CpG n=16;

NcMP/CpG n=18. Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group; detection limit (DL) is indicated by a horizontal line; (* $p<0.05$)

Protected mice present lower IFN-γ production following *N. caninum*-specific cell stimulation

IFN-γ is the hallmark cytokine of the Th1-type immune response and its paramount importance for the host resistance against neosporosis has been shown in different studies[29,30]. Since half of the $p40^{-/-}$ immunized mice were protected upon i.g. challenge with *N. caninum*, and an IgG1/IgG2a ratio < 1 was observed in spite of IL-12 prominent role in Th1 polarization, we assessed if an increase in parasite-specific IFN-γ production elicited by the immunization protocol could be mediating the observed protection. Therefore, splenocytes from one-week infected immunized and control animals were *in vitro* stimulated for 3 days with NcS. As shown in Figure 6-4a IFN-γ levels in the cell culture supernatants were not significantly different among the assessed groups. Nevertheless, in this analysis, non-colonized immunized mice presented lower IFN-γ concentrations in comparison with similarly immunized but colonized animals. In fact, when we plotted (Figure 6-4c) the concentrations of IFN-γ in the culture supernatants of splenocytes from non-colonized versus colonized mice in the immunized group we observed a lower production of this cytokine in the former. Additionally, and as shown in Figure 6-4b, no significant differences were observed in the production of IL-4, the prototypic Th2 cytokine, associated with host susceptibility to neosporosis[51,52].

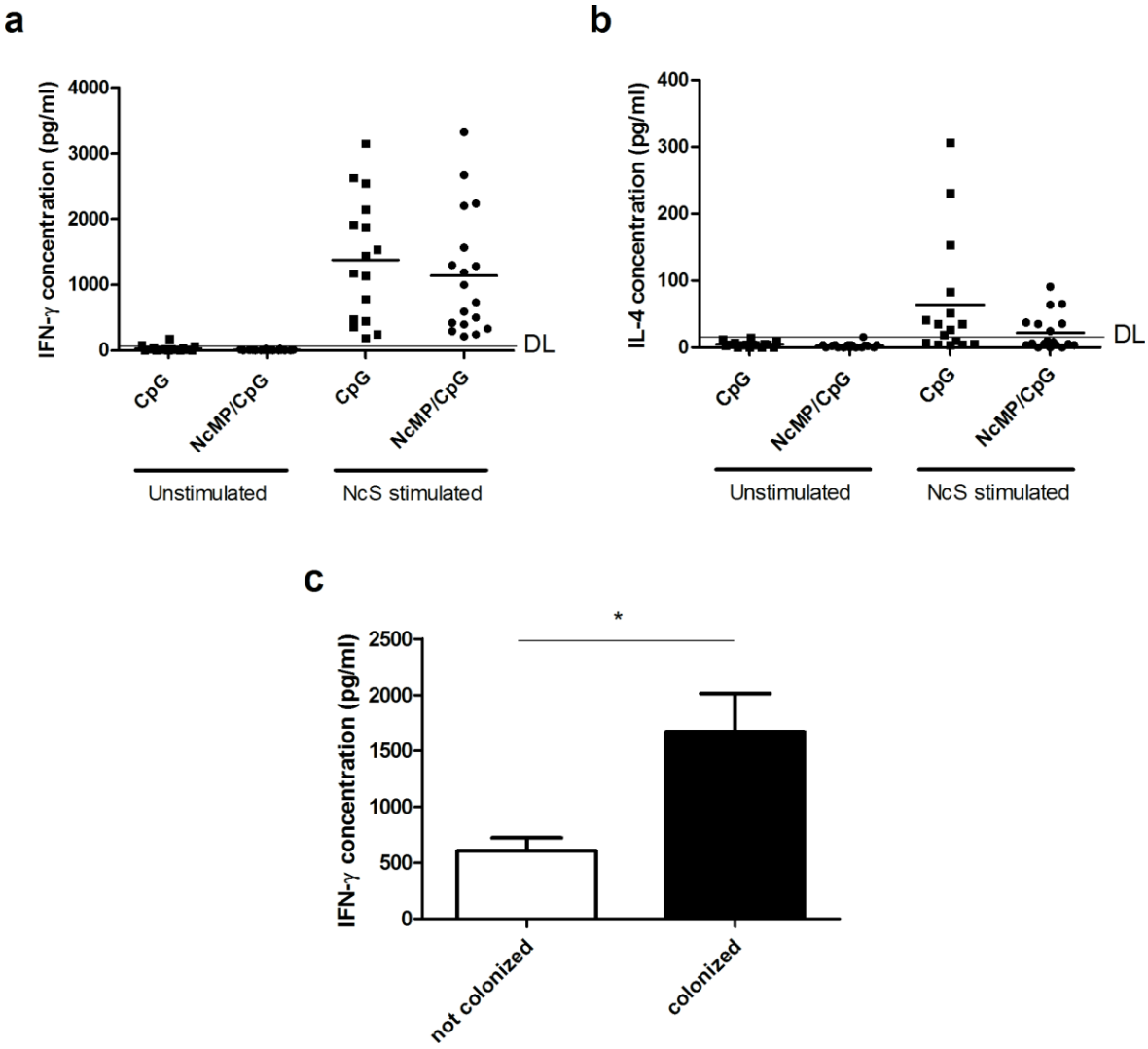

Figure 6-4 - IFN-γ (a) and IL-4 (b) concentration in the supernatants of splenocytes cell cultures unstimulated or stimulated for 3 days with *N. caninum* sonicates (NcS). Cells were isolated from the spleens of mice one week upon i.g. challenge with 5×10^7 *N. caninum* tachyzoites that have been previously immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG). (c) Separation and comparison of data regarding IFN-γ concentration between colonized and uncolonized mice in the NcMP/CpG group. Results are from pooled data of two independent experiments and stimulated conditions were analyzed in duplicate for each mouse. Total number of mice per group: CpG n=16; NcMP/CpG n=18. Horizontal lines in scatter plots correspond to the mean value in each group while bars in column graphs represent mean plus one SEM. DL - below detection limit of the kit (15 pg/mL); (* $p<0.05$).

Reduced parasitic burden in immunized p40$^{-/-}$ mice infected i.p. with *N. caninum*

Since no detectable parasite DNA was found in immunized mice following the i.g. parasitic challenge in the absence of increased IFN-γ production, other protective mechanisms could account for the observed protection. As we have previously shown that parasite-specific intestinal IgA produced following i.n. immunization with NcMP and CpG could agglutinate *N. caninum* tachyzoites[46] (chapter 4), this immunoglobulin could be mediating protection in the immunized p40$^{-/-}$ mice by preventing host infection. Therefore, in order to rule out the possible host protective effect of intestinal IgA *in vivo,* we i.p infected similarly immunized mice and sham-immunized controls with 1 × 10^4 *N. caninum* tachyzoites. One week following infection we analyzed by qPCR the brain parasitic burden. As shown in Figure 6-5, mice receiving NcMP plus CpG presented a clear reduction in parasite burden following i.p. challenge when compared with sham-immunized controls. In addition, no *N. caninum* DNA was detected in 6 out of 14 immunized mice once again indicating that this immunization strategy elicited a host protective immune response even when the i.p. infection route was used in p40$^{-/-}$ mice.

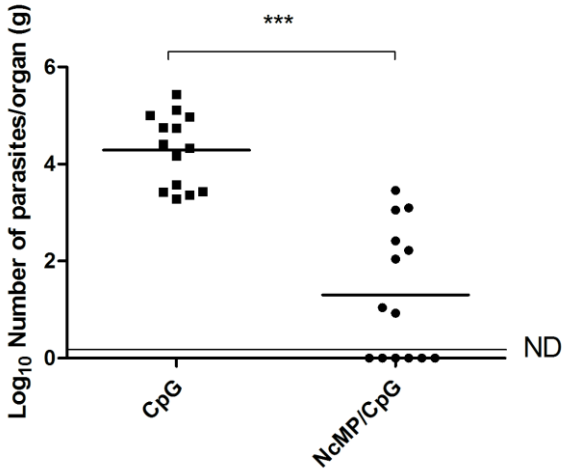

Figure 6-5 - Parasitic load assessed by qPCR one week upon i.p. challenge with 1 × 10^4 *N. caninum* tachyzoites in the brain of mice previously immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG). Data is presented as $\log_{10}$ of the number of parasites by gram of organ. Results are from pooled data of two independent experiments. Total number of mice per group: CpG n=14; NcMP/CpG n=14. Each dot represents an individual mouse. Horizontal lines correspond

to the mean value in each group; detection limit (DL) is indicated by a horizontal line. (*** p<0.001)

Passive immunization slightly reduces parasite burden in i.p. infected p40$^{-/-}$ mice

Parasite-specific IgG elicited following i.n. immunization of WT mice was capable of opsonizing *N. caninum* tachyzoites and reduce parasite survival in *in vitro* infected macrophages[46] (chapter 4). Therefore, it was conceivable that parasite-specific IgG in the serum of immunized p40$^{-/-}$ mice could contribute to the observed protection in i.p. infected mice. In order to confirm the existence of this host protective mechanism, we purified IgG from the serum of previously i.n. immunized (IgG-NcMP) and sham-immunized (IgG-CpG) mice and respectively transferred each IgG fraction into recipient naïve p40$^{-/-}$ mice. Twelve hours following passive immunization all mice were i.p. infected with 1 × 10^4 *N. caninum* tachyzoites and the parasite burden analyzed one week after infection by qPCR. As shown in Figure 6-6, a small reduction in parasitic burden could be observed between mice receiving control IgG-CpG and mice receiving the IgG-NcMP fraction although not reaching statistical significance.

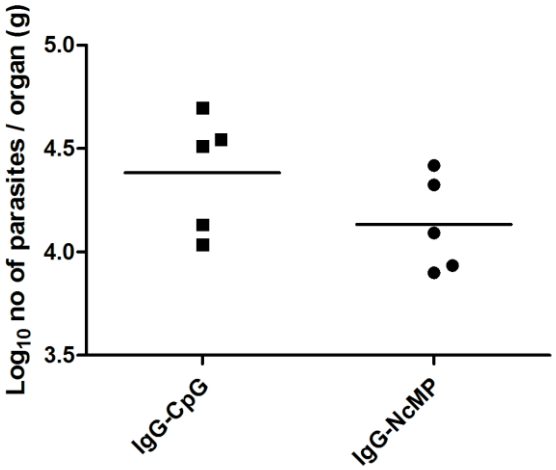

Figure 6-6 - Parasitic load assessed by qPCR one week upon i.p. challenge with 1 × 10^4 *N. caninum* tachyzoites in the brain of mice passively immunized with control IgG purified form the serum of sham-immunized mice (IgG-CpG) or with IgG isolated from the serum of previously immunized mice (IgG-NcMP). Data is presented as $\log_{10}$ of the number of parasites by gram of organ. Results are from pooled data of two independent

experiments. Total number of mice per group: CpG n=5; NcMP/CpG n=5. Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group

Immunized p40$^{-/-}$ mice infected i.p. did not present increase IFN-γ production

Since a protective effect of serum parasite-specific IgG was not evident, we also analyzed IFN-γ production by spleen CD4^{+} and CD8^{+} T cells in immunized p40$^{-/-}$ mice infected i.p. As shown in Figure 6-7a, no significant differences in CD4^{+} and CD8^{+} T cell numbers were observed between sham- and immunized mouse groups. The analysis of the frequency of CD4^{+} and CD8^{+} T cells producing IFN-γ (Fig 6-7b) revealed a higher proportion of CD8^{+}IFN-γ^{+} T cells in comparison with the proportions observed for CD4^{+}IFN-γ^{+} T cells. However, no significant differences in the frequencies (Figure 6-7b) and numbers (Figure 6-7c) of CD4^{+} and CD8^{+} T cells producing IFN-γ were observed between the two groups. As could be expected, *in vitro* splenocyte cell cultures stimulated with NcS did not revealed any significant difference regarding the production IFN-γ or IL-4 between cells of immunized and sham-immunized groups (Figure 6-7d).

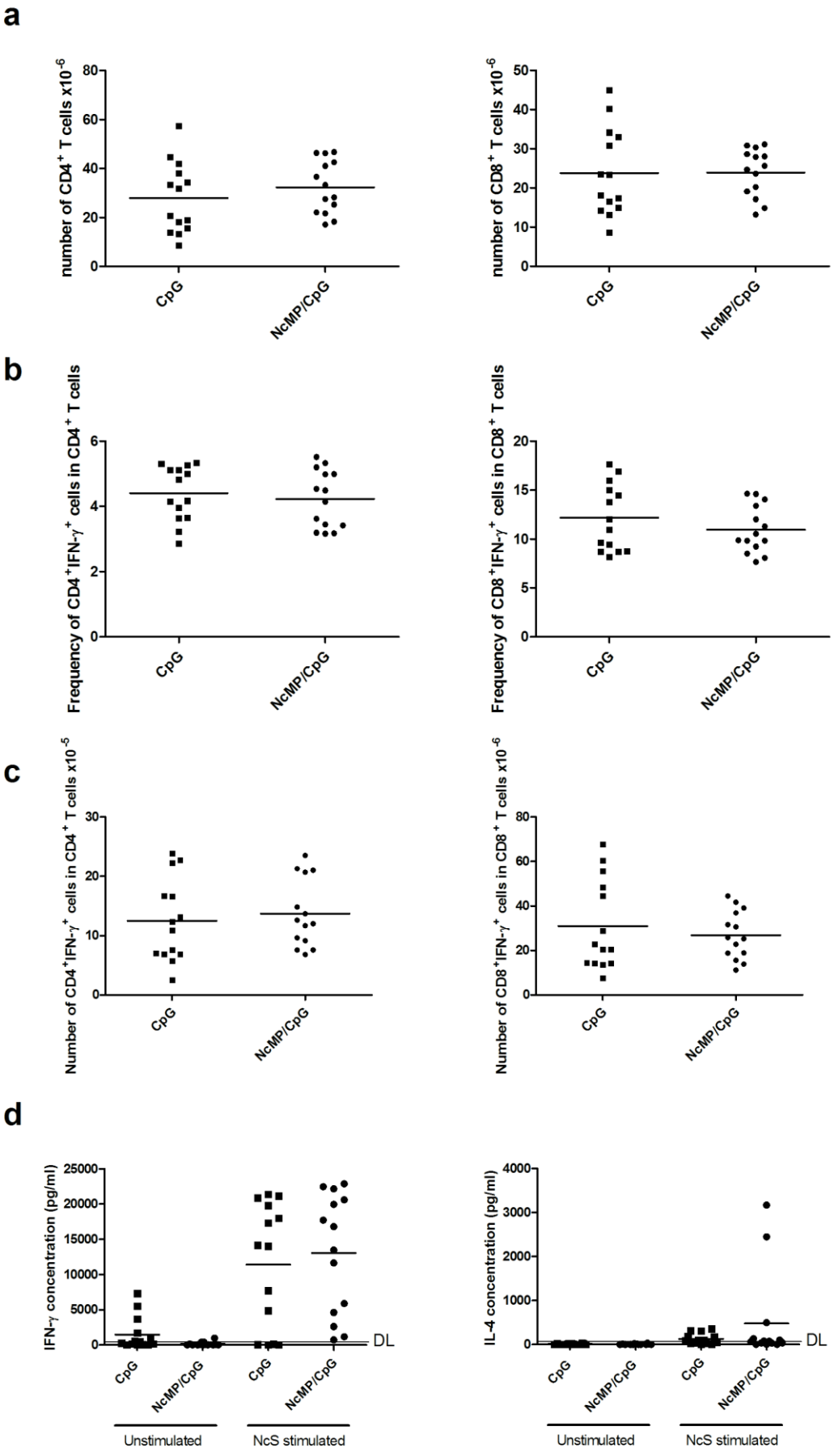
a
number of CD4+ T cells x10-6
80
60
40
20
0
CpG
NcMP/CpG
number of CD8+ T cells x10-6
50
40
30
20
10
0
CpG
NcMP/CpG
b
Frequency of CD4+IFN-γ+ cells in CD4+ T cells
6
4
2
0
CpG
NcMP/CpG
Frequency of CD8+IFN-γ+ cells in CD8+ T cells
20
15
10
5
0
CpG
NcMP/CpG
c
Number of CD4+IFN-γ+ cells in CD4+ T cells x10-5
30
20
10
0
CpG
NcMP/CpG
Number of CD8+IFN-γ+ cells in CD8+ T cells x10-6
80
60
40
20
0
CpG
NcMP/CpG
d
IFN-γ concentration (pg/ml)
25000
20000
15000
10000
5000
0
DL
CpG
NcMP/CpG
CpG
NcMP/CpG
Unstimulated
NcS stimulated
IL-4 concentration (pg/ml)
4000
3000
2000
1000
0
DL
CpG
NcMP/CpG
CpG
NcMP/CpG
Unstimulated
NcS stimulated

Figure 6-7 - Total number of splenic $CD8^+$ and $CD4^+$ T cells (a) and frequencies (b) or numbers (c) of splenic $CD8^+IFN\text{-}\gamma^+$ or $CD4^+$ $IFN\text{-}\gamma^+$ T cells, detected one week after i.p. challenge with 1×10^4 *N. caninum* tachyzoites in mice previously immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG). (d) IFN-γ and IL-4 concentration in the supernatants of splenocytes cell cultures unstimulated or stimulated for 3 days with *N. caninum* sonicates (NcS). Results correspond to pooled data of two independent experiments. Cytokine analysis in cell culture supernatants in stimulated conditions were analyzed in duplicate for each mouse. CpG n=14; NcMP/CpG n=14. Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group. DL - below detection limit of the kit (15 pg/mL)

DISCUSSION

In this work we have used an IL-12-deficient mouse model in order to further study the host protective mechanisms elicited by i.n. immunization with NcMP and CpG adjuvant. In our previous work we have demonstrated that this i.n. immunization strategy conferred long-term protection against i.g. established neosporosis in the absence of a marked Th1-type immune response in C57Bl/6 WT mice[46] (chapter 4 and 5). Although the IL-12/IFN-y axis has been shown to be essential for host resistance against *N. caninum* infection[27-32], our results hinted to the existence of a protective mechanism that could be mediated by intestinal parasite-agglutinating IgA and serum parasite-opsonizing IgG. In this model, the undetectable parasite colonization observed in the majority of immunized animals along with the absence of increased IFN-γ production could indicate that a humoral mediated mechanism prevented host infection[46] (chapter 4 and 5).

Analysis of the humoral response elicited in the immunized $p40^{-/-}$ mice revealed that these presented similar levels of parasite-specific intestinal IgA and serum IgG in comparison with those previously reported in similarly immunized WT mice[46] (chapter 4). Although $p40^{-/-}$ mice were not described has having a compromised humoral response[53], to our knowledge this is the first time that IgA titres have been assessed in the mucosa of IL-12-deficient mice following mucosal immunization showing that absence of this cytokine did not significantly impact IgA production. Surprisingly, taking into consideration the IL-12-deficient phenotype, but in accordance with our previous observations[46] (chapter 4 and 5), an IgG1/IgG2a ratio < 1 was detected in the serum of immunized $p40^{-/-}$ mice. Due to the importance of IFN-γ in IgG2a production[50], this result indicates that a small bias towards a Th1-type immune response was induced. A likely candidate that could explain this observation due to its known Th1 immunostimulatory properties is CpG[54-56] that can also lead to the production of IL-18[57], an important cytokine inducing IFN-γ production by natural killer and T cells[58,59]. In fact, IL-18 has been shown in previous studies to mediate IFN-γ production in IL-12-deficient mice[60]. Nevertheless, and to our knowledge, CpG has never been used as an adjuvant in IL-12-deficient mice and therefore it would be worth assessing in future studies if

this adjuvant could indeed also have Th1-type immunostimulatory proprieties in the absence of IL-12, a key Th1-inducing cytokine induced by this adjuvant[54-56].

In accordance with the IgG1/IgG2a ratio, following i.g. infection of immunized p40$^{-/-}$ mice and although IFN-γ was detected in both experimental groups, no significant differences were observed in the production of this cytokine between immunized and sham-immunized controls. In this regard, IFN-γ production in IL-12-deficient mice has already been described following infection with different protozoa[60-62] in a process dependent on IL-18[60]. On the other hand, and once again in accordance with our previous results[46] (chapter 4), very low levels of IL-4 were detected in both immunized and control mice splenocyte cultures. Additionally, although presenting a compromised Th1-type immune response, infection of IL-12-deficient mice without increase IL-4 production has been reported in previous studies[61].

Following the assessment of the elicited immune response, we infected immunized p40$^{-/-}$ mice i.g., in order to further understand if protection conferred by the i.n. immunization protocol used here could indeed be attained in the absence of a strong Th1-type immune response. Parasitic burden analysis by qPCR revealed that protection could indeed be achieved and that protected p40$^{-/-}$ mice did not present detectable parasite colonization levels, in agreement with the protection profile observed in our previous studies[46] (chapter 4 and 5). Protection against other pathogens[61,63,64], including the apicomplexa *Plasmodium berghei*[62], has already been shown to be possible in the absence of IL-12. Still, to our knowledge, this is the first time that protection against *N. caninum* infection through immunization has been shown in a mouse model with a compromised Th1-type immune response. Although a significant number of mice could be protected following i.g. infection, when successful parasite colonization did occur, no significant differences were observed between the mean parasitic burdens of immunized and sham-immunized controls. In addition, immunized mice with undetectable parasitic DNA presented a much lower IFN-γ production in comparison with immunized and infected mice. Both results support our previously proposed model[46] (chapter 4), whereby protection induced by i.n. immunization with NcMP and CpG could be mediated through intestinal parasite-agglutinating IgA that in turn could prevent host infection, leading to undetectable parasite DNA

and reduced antigen stimulation, resulting in lower cytokine production following antigen recall. Nevertheless, future analysis will be needed to fully confirm this hypothesis. In this regard, immunization of intestinal IgA-deficient mice would help ascertain if parasite-agglutinating IgA in the intestinal mucosa could be mediating host protection.

In order to further explore the possible host protective effect of parasite-specific intestinal IgA elicited following i.n. immunization, we infected i.p. $p40^{-/-}$ mice immunized i.n., therefore bypassing the intestinal mucosal barrier. Surprisingly, immunized mice still presented a reduced number of colonized animals and a reduction in the overall colonization levels in comparison with sham-immunized controls. Furthermore, and in contrast with i.g. infected mice, following i.p. infection immunized mice presented lower parasitic burdens than sham-immunized controls. Despite the relatively high levels of IFN-γ produced by splenocytes *in vitro* following parasite-specific antigenic recall, once again no major differences in IFN-γ production was observed between immunized and sham-immunized controls. Additionally, and in contrast with the observations made in i.g. infected immunized mice, non-colonized and immunized $p40^{-/-}$ mice infected i.p. did not present reduced IFN-γ production. However, in this case, and due to the used infection route, antigenic stimulation should have occurred in all mice regardless of their colonization status. Furthermore, although affording a slight reduction in parasitic burdens, IgG transfer by passive immunization did not recapitulate the protection observed in i.p. infected immunized $p40^{-/-}$ mice. This result indicates that despite the previously shown *in vitro* host protective capacity of elicited parasite-specific IgG[46] (chapter 4), these could at best marginally contribute to the observed protection. Nevertheless, and since IgG specific for some membrane proteins of *N. caninum* have been shown *in vitro* to be capable of preventing parasite infection of host cells[34-40], it would be worth confirming if passive immunization trough IgG transfer could indeed confer some degree of protection *in vivo*, a point that has never been assessed following *N. caninum* infection. Still, the protection observed in i.p. infected immunized $p40^{-/-}$ mice indicates that other mechanism besides the possible host protective role of IgA should be elicited following immunization. Since IgG appears to have a marginally protective effect, a cell-mediated immune response is most likely responsible for

the protection observed in i.p. infected mice. In this regard, consecutive priming with *P. berghei* has shown that an early and strong production of IFN-γ in IL-12-deficient mice can be induced, which could confer protection against an otherwise lethal challenge[62]. It could be speculated that a similar priming of a Th1-type immune response could be achieved following i.n. immunization of $p40^{-/-}$ mice. In this sense, an early production of IFN-γ in immunized mice could allow a quicker control of parasite dissemination in contrast with sham-immunized controls. Although no difference between groups was found regarding IFN-γ production one week after infection, since higher IFN-γ production was observed in similarly immunized WT mice prior to infection (chapter 5), it would be of interest to see if a similar response could be observed in immunized and uninfected $p40^{-/-}$ mice. Another point that should be taken into consideration is the difference in cellular composition and elicited immune response in the intestinal mucosa and in the peritoneal cavity. Due to the widespread use of i.p. infection in murine models of *N. caninum* infection it should be kept in mind that the protective effect elicited trough immunization can be dependent on the infection route used. In this regard, the i.g. infection route used here more closely mimics the natural route taken by the parasite during horizontally transmitted neosporosis.

Altogether, the obtained results show that i.n. immunization with NcMP and CpG conferred protection to both i.g. and i.p. infected mice harboring a mutation compromising Th1-type immunity. The results showed here support a model in which protection against *N. caninum* could be mediated by parasite-specific IgA preventing host infection. Nevertheless different protective mechanism elicited by immunization might account for the observed results as i.g. versus i.p. infection evidenced that the route of infection used can have a great impact in determining the protective effect of immunization against this protozoan.

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Chapter 7

Intranasal immunization with *Neospora caninum* membrane antigens and CpG in cattle induces a parasite-specific humoral and cell-mediated immune response

Intranasal immunization with *Neospora caninum* membrane antigens and CpG in cattle induces a parasite-specific humoral and cell-mediated immune response

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ABSTRACT

Successful or practical control options to manage or prevent infection with the abortive intracellular cattle pathogen *Neospora caninum* are currently unavailable in spite of the high economic losses associated with this parasite infection. In this regard, we have previously demonstrated the host protective effect of an intranasal immunization protocol using *N. caninum* membrane proteins in different mice models following intragastric or intraperitoneal challenge. In this work we evaluated the immunogenicity and elicited immune response following intranasal immunization with *N. caninum* membrane proteins and cattle-optimized CpG adjuvant in Holstein calves. Three weeks following a single boost immunization animals presented elevated parasite-specific serum IgG and IgA titres. In addition, *in vitro* cell cultures of peripheral blood mononuclear cells collected at the same time point presented a significant increase in interferon-γ production when co-cultured with *N. caninum* tachyzoites in comparison with similarly stimulated cells obtain from sham-immunized control animals. Altogether, these results demonstrate the immunogenicity of our intranasal immunization protocol and prove that this strategy can induce a humoral and cellular parasite-specific immune response in immunized calves.

INTRODUCTION

Although initially described in dogs[1], the apicomplexa parasite *Neospora caninum* has in the last decades gain notoriety as an abortive pathogen in cattle[2,3]. *N. caninum* is one of the most successful vertically transmitted parasites in cattle [2] and although, in most instances, *in utero* infection leads to the birth of a congenially infected but otherwise healthy calf[4-6], infected dams present a considerable increase in abortion rates[7-13]. This reproductive impairment has been recognized and associated with severe economic losses in beef and dairy cattle livestock farms leading to worldwide losses estimated above 1.3 billion dollars per year[14]. Although vaccination is regarded as the preferable option to control this parasitic infection[15] no commercial vaccine is nowadays available[16,17]. Nevertheless, a great effort to develop an effective immunization strategy that could block vertical transmission or prevent cattle infection altogether has been undertaken in the last decades as evidenced by the considerable number of experimental immunization studies[16-18].

Although not normally considered a natural host for *N. caninum*, for several years mice have been established in laboratory conditions as a suitable model to study neosporosis[19-21]. In this regard, mice have been extensively used to explore and characterized *N. caninum* host-parasite relationship as well as to assess the protective effect of several immunization protocols[16-18]. Still, despite the usefulness of mice for large scale screening of promising vaccination strategies, confirmation in cattle of the immunogenicity and host protective effect of promising immunization protocols is essential. In this regard, most immunization protocols assessed in cattle, although reporting encouraging results in the prevention of abortion and vertical transmission, have achieved this protection with the use of live[22] and attenuated[23-26] parasite strains which present considerable drawbacks of production, safety and stability[26,27]. In contrast, cattle immunization with sub-unit vaccines has been performed in very limited occasions and with mixed protection outcomes despite promising results of several protocols in mouse models, and their advantages for mass vaccination implementation[28-30]. Furthermore, and in spite of the fact that in horizontal transmitted neosporosis *N. caninum* infects the host through the gastrointestinal mucosa[2], the use of a mucosal vaccination

strategy has never been attempted in cattle despite the recent encouraging results obtained in mice models with intranasal (i.n.) immunization protocols[31-33] (chapter 4). We have recently demonstrate the protective effect of an i.n. immunization protocol using *N. caninum* membrane proteins (NcMP) and CpG adjuvant against intragastrically (i.g.)-established neosporosis in different mouse models[33] (chapter 4, 5 and 6). Nevertheless the immunogenicity of our developed formulation has never been assessed in cattle. As such, in this study we i.n. immunized Holstein calves with NcMP and a cattle-optimized CpG and evaluated the induction of humoral and cellular parasite specific immune responses.

MATERIALS AND METHODS

Animals

Nine month-old *N. caninum* and Bovine viral diarrhea virus seronegative female Holstein calves were used in experimental immunization protocols. Animals were kept at the Large Animal Farm of Instituto de Ciências Biomédicas Abel Salazar (ICBAS) throughout the experimental procedures. Experiments were approved by the institutional board responsible for animal welfare (ORBEA) at ICBAS.

Parasites

N. caninum tachyzoites (Nc1 isolate) were kept by serial passages in VERO cells cultures, maintained in Minimal Essential Medium containing Earle's salts (Sigma, St. Louis, USA), supplemented with 10% fetal calf serum (Biowest, Nuaillé, France), L-Glutamine (2 mM), Penicillin (200 IU/ml) and Streptomycin (200 µg/ml) (all from Sigma), in a humidified atmosphere with 5% CO_2 at 37 ^{0}C. Tachyzoites were maintained until destruction of 80% of the host cell monolayer and were isolated as previously described[34]. Briefly, free parasites and adherent cells were recovered using a cell scraper and centrifuged at 1 500 × g for 15 min. The pellet was passed through a 25 G needle and then washed three times in PBS by centrifugation at 1 500 × g for 15 min. The resulting pellet was resuspended and passed through a PD-10 desalting column, containing Sephadex™ G-25M (GE Healthcare, Freiburg, Germany). Tachyzoites concentration was determined in a haemocytometer.

Preparation of whole tachyzoite lysates and cell-membrane extracts

N. caninum membrane proteins (NcMP) were extracted using a modification of a previously described method[35,36]. Briefly, free tachyzoites were resuspended in PBS containing 0.75% triton X-114 (Sigma), incubated 10 min on ice and centrifuged at 10 000 × g for 30 min at 4 ^{0}C and the supernatant was recovered and placed in a water bath at 30 ^{0}C during 3 min. The procedure was repeated and the supernatant was centrifuged at 1 000 × g for 3 min at room temperature.

The aqueous phase was discarded and the NcMP were precipitated with the addition of absolute ethanol, vortexed vigorously during 15 sec and incubated 1 h on ice. The samples were centrifuged at 12 000 × g for 20 min at 4 ^{0}C and the resulting pellet was dried, resuspended in PBS and stored at -20 ^{0}C. NcMP quantification was performed by using the Lowry protein assay. Sodium dodecyl sulphate polyacrylamide gel electrophoresis followed by silver nitrate staining was performed following each protein extraction in order to determine and confirm the protein migration profile.

Immunizations and blood sample collection

Nine-month old calves were distribution into 2 groups and immunized i.n. twice with three weeks interval with 500 µl of PBS containing, 400 µg of the previously described[37] bovine optimized CpG ODN 2135 (TCGTCGTTTCTCGTTTTGTCGTT, obtained from Invitrogen, MA, USA) (CpG group, n=4), or PBS containing 500 µg of NcMP plus 400 µg of CpG ODN 2135 (NcMP/CpG group, n=4). i.n. application was performed by administering 250 µl in each nostril with a syringe coupled with an intranasal dispersion unit (Rispoval, Pfizer, NY, USA). The animals' head was contained in an upright position for 30s to prevent rejection of the administered formulation. Blood samples were collected by tail venipuncture 3 weeks after the boost immunization for humoral and cell mediated immune response analysis.

Antibody detection

Serum samples obtained from blood collected 3 weeks after the boost immunization were used to evaluate by ELISA the presence of NcMP-specific IgG1, IgG2 and IgA antibodies. Briefly, 96-well plates (Maxisorp, Nunc, Denmark) were coated overnight at 4 ^{0}C with NcMP diluted in PBS at a concentration of 5 µg/ml. All the wells were saturated with 2% bovine serum albumin (BSA) (Sigma) in TST buffer (150 mM NaCl, 10 mM EDTA and 0.05% Tween 20, pH=8) for 1 h. Serum samples were serially diluted in 1% BSA TST buffer and incubated for 1 h, followed by washing and addition of alkaline phospatase-coupled polyclonal sheep anti-bovine IgG1, IgG2 or IgA (all from Bethyl, TX, USA) and incubation for 1 h. After washing, the specifically bound antibodies were detected by the development

with the substrate solution of p-nitrophenyl phosphate (Sigma) and the reaction was stopped with the addition of 0.1 M EDTA, pH=8 solution. The absorbance was measured at 405 nm, subtracting for each well the value for the absorbance at 570 nm. The antibody titres were expressed as the $\log_{10}$ value of the reciprocal highest dilution with an absorbance higher than the value of the control (no serum added).

In vitro cell cultures and cytokine detection

Blood mononuclear cells (PBMC) were collected from blood samples obtained 3 weeks after the boost immunization to assess parasite-specific cytokine production in peripheral blood. Briefly, collected blood was diluted and layered over a solution of polysucrose-sodium ditrizoate (Histopaque 1077, Sigma) and centrifuged at 800 × g for 20 min at room temperature. Recovered cells in the blood Histopaque interface were washed, twice and suspended in RPMI-1640 (Sigma), supplemented with 10% fetal calf serum (Biowest), HEPES (10 mM), Penicillin (200 IU/ml) and Streptomycin (200 µg/ml) (all from Sigma), and β-mercaptoethanol (0.1 µM, Merk, Darmstadt, Germany). Cells were plated in triplicates per animal in round bottom 96-well plates (5×10^5 cells/well) (Nunc) and stimulated with 200 Gy gamma-irradiated *N. caninum* tachyzoites (Gammacell1000Elite irradiator, Nordion International, Inc., Ottawa, Canada; $1x10^6$ tachyzoites/well) for 5 days at 37 ºC and 5% CO_2. The concentration of IFN-γ and IL-4 in cell culture supernatants was assessed by ELISA using the Bovine IFN-γ or IL-4 screening set kit (Thermo scientific, IL, USA), respectively and according to manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad prism, Version 5.0 (GraphPad Software, Inc., La Jolla, CA). Column graphs are represented showing the mean plus one standard error of the mean (SEM). Statistical analysis between groups was performed by using student's *t*-test.

RESULTS

NcMP/CpG immunized calves present antigen-specific serum IgG and IgA

In previous studies[33] (Chapter 4 and 5), i.n. immunization in C57Bl/6 WT mice with NcMP and CpG induced the production of parasite-specific mucosal IgA and serum IgG. In order to assess if a similar immunization procedure in calves could also induce an antigen-specific humoral immune response, we screen immunized calves serum samples obtained 3 weeks after the last immunization for the presence of NcMP-specific IgA and IgG. As shown in Figure 7-1a, immunized calves presented an antigen-specific IgG response with mixed production of IgG1 and IgG2, while no detectable antigen-specific IgG titres were observed in sham-immunized controls, as expected. Since production of IgG1 and IgG2 has also been shown to be associated in cattle with an underlying Th2- and Th1-type immune response, respectively[38,39], we determined the ratio between the production of these two immunoglobulins as an indicator of the elicited cellular immune response in immunized animals. Using the mean titre values for each IgG isotype, we obtain a calculated IgG1/IgG2a ratio < 1 indicating that a small bias towards a Th1-type was achieved in i.n. immunized calves.

Since the induction of IgA production is considered a hallmark of successful induction of a mucosal immune response[40-43], and since direct analysis of the small intestine IgA production was impossible under the described experimental conditions, we determined the existence of NcMP-specific IgA in the serum of immunized calves as an indicator of mucosal immune response induction. As shown in Figure 7-1b, three weeks after the last immunization NcMP/CpG immunized calves presented elevated antigen-specific IgA titres. Once again and as expected, no detectable NcMP-specific IgA titres were found in sham-immunized controls. Altogether, these results indicate that i.n. immunization of calves with NcMP and CpG is capable of inducing a parasite-specific humoral response

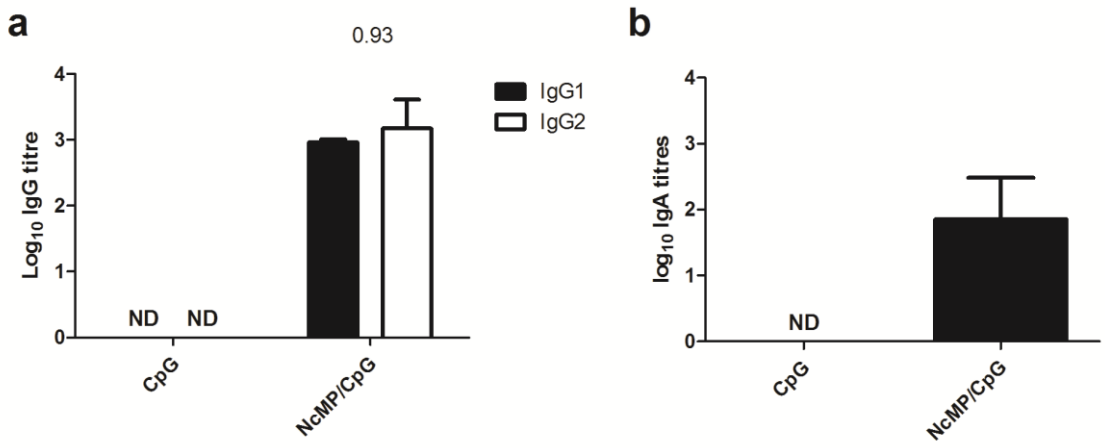

Figure 7-1 - Serum titres of *N. caninum* membrane proteins (NcMP)-specific IgG (a) (IgG1 - closed bar and IgG2 - open bar) and IgA (b). Immunoglobulin titres were determined by ELISA in serum samples collected 3 weeks upon boost immunization from calves immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG). Data is presented as log_{10} of the antibody titres, as indicated. Total number of calves per group: CpG n=4; NcMP/CpG n=4. Numbers above bars represent the IgG1/IgG2 ratio calculated with the mean log_{10} titres of each respective IgG isotype. Graphs are presented with mean plus one SEM; ND – not detected

PBMC from i.n. immunized calves present increased parasite-specific IFN-γ production

To evaluate if i.n. immunization with NcMP/CpG could induce a cell-mediated immune response in immunized calves we collected PBMC 3 weeks after the boost immunization. These cells were co-culture *in vitro* with irradiated *N. caninum* tachyzoites for 5 days after which we analyzed the cell culture supernatants for the presence of IFN-γ and IL-4 since these cytokines have been implied in host resistance and susceptibility to *N. caninum* infection, respectively[44,45]. As shown in Fig 7-2, and as indicated by the previously calculated IgG1/IgG2a ratio, *N. caninum* stimulated PBMC obtained from immunized calves presented higher IFN-γ production levels when compared with similarly stimulated PBMC collected from sham-immunized controls. In sharp contrast, undetectable levels of IL-4 were observed in similarly stimulated cells from both immunized and sham-immunized calves (data not shown)

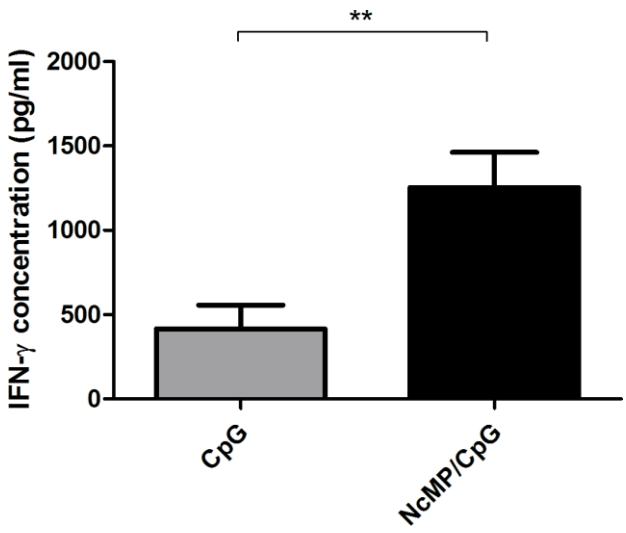

Figure 7-2 - IFN-γ concentration in the supernatants of PBMC cell cultures stimulated for 5 days with $1x10^6$ gamma-irradiated *N. caninum* tachyzoites. Cells were isolated from blood samples collected 3 weeks upon boost immunization from calves immunized twice i.n. with NcMP and CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG). Total number of calves per group: CpG n=4; NcMP/CpG n=4. Graphs are presented with mean plus one SEM (** $p<0.01$).

DISCUSSION

Although economic losses due to *N. caninum* related abortions constitute a significant burden in affected cattle livestock farms[14], cost-effective control options to manage this parasitic infection are still not available[46]. Economic models have indicated that a successful vaccination protocol that could prevent primary infection or the parasite's vertical transmission constitute the best strategy to control neosporosis[15,27], but with the exception of the now withdrawn and largely unsuccessful commercial vaccine Bovilis® Neoguard[47,48], no other vaccine has ever made it into the market. Nevertheless, in the last decades several promising immunization strategies against neosporosis have been tested, mainly in mice models of neosporosis[16,17]. Still, and due to obvious limitations of performing experimental work with large animals, the evaluation of promising immunization protocols in cattle, the final target species, although necessary and mandatory has been limited.

In previous works we have demonstrated that i.n. immunization with NcMP and CpG as adjuvant could confer long-term protection against intragastrically established murine neosporosis[33] (Chapter 4 and 5), making this protocol a promising strategy to induce a cattle protective immune response against neosporosis. Therefore, in this work we have assessed the immunogenicity and the elicited immune response in similarly i.n. immunized Holstein calves using a cattle-optimized CpG[37]. Since the natural route of infection in horizontally transmitted neosporosis is through the gastrointestinal tract, the induction of a parasite-specific mucosal immune response trough vaccination could be a determining factor to achieve host protection against this parasite. Still, and to best of our knowledge, this work reports the results of the first mucosal immunization protocol that has ever been used in cattle to induce a host protective immune response directed against *N. caninum*. In addition, and although CpG has already been employed in combination with other adjuvants in the formulation of experimental vaccines in cattle[49-53], this is also the first time that CpG has been used in this species in a mucosal immunization protocol, despite its known powerful mucosal adjuvant properties already described in both human and mouse models[54,55].

Taking into account that i.n. immunization with NcMP in mice induced the production of parasite-agglutinating intestinal IgA and opsonizing serum IgG antibodies that could be active mediators in the reported protection[33] (Chapter 4), we wondered if similar immunization in calves could also induce a comparable parasite-specific humoral response. Isotype analysis of the humoral response in the serum of immunized calves revealed the presence of parasite-specific IgA and IgG indicating that this protocol was successful at inducing a humoral parasite-specific response. Since IgA is regard as a hallmark of the induction of successful mucosal immunity[40-43], this preliminary analysis suggests that our immunization protocol should have successfully induced a parasite-specific mucosal immune response. Still, despite the fact that serum IgA has already been used in previous studies to evaluate an characterize the effectiveness of i.n. immunization in cattle[56], serum IgA levels have been known to not always give the best readout regarding mucosal production of this immunoglobulin[41,57]. Therefore, and since parasite-agglutinating intestinal IgA elicited in similarly i.n. immunized mice could be a major mediator of the previously observed protection[33] (chapter 4), it would be worth assessing in future studies if parasite-specific IgA is also present in the intestinal mucosa or in fecal contents of i.n. immunized calves. This analysis would also confirm if an intestinal mucosal immune response could be induced following i.n. immunization in calves, a point never assessed in cattle. In this regard, previous studies indicate that i.n. immunization, although capable of inducing both mucosal and systemic immune response to target antigens in mice, induced somewhat weak responses in the intestinal mucosa[41,58], despite evidence of cell trafficking between the two mucosas following i.n. immunization in mice[59,60]. Since in i.n. immunized sheep, antigen-specific IgA has been found in fecal contents following immunization[61], a similar cellular trafficking between the nasal and intestinal associated lymphoid tissues should also exist in ruminants.

Isotype analysis of the elicited parasite-specific serum IgG response revealed an IgG1/gG2 ratio < 1 indicating that a small bias towards a Th1-type immune response was achieved. This result is consistent with the known Th1-type immunomodulatory activity of CpG[58,62,63], that has also been previously reported in cattle immunized with CpG containing formulations[49-52]. Furthermore, a IgG1/IgG2a ratio < 1 has been found in similarly i.n. immunized mice with NcMP

and CpG[33] (chapter 4), indicating that a comparable immune response was achieved in immunized calves. On the other hand, and since similarly i.n. immunized mice present serum parasite-opsonizing IgG that could reduce parasite survival when in co-culture with macrophages[33] (chapter 4), it would also be worth assessing if the elicited IgG in the serum of immunized cattle could also mediate a similar and possibly host-protective mechanism. In this regard, and although our preliminary results following passive immunization with parasite-specific IgG in IL-12p40$^{-/-}$ revealed a marginal protective effect of this immunoglobulins *in vivo* (chapter 6), a possible host-protective effect of serum IgG against *N. caninum* infection has never been explored in cattle.

In line with the humoral analysis indication that in immunized calves a Th1-biased immune response was achieved, and taking into account that IFN-γ is widely known for its paramount importance in host resistance against *N. caninum* infection[44,45], cytokine production analysis in PBMC following parasite-specific stimulation revealed that cells obtained from immunized calves presented elevated levels of IFN-γ in comparison with similarly stimulated cells obtain from sham-immunized controls. In sharp contrast, the Th-2 associated cytokine IL-4, linked with host susceptibility to this parasite infection[44,45] was undetected in similarly stimulated PBMC from both immunized and sham-immunized controls. These results are in agreement with previous results obtained in immunized mice where parasite-specific stimulation of MLN and spleen cells also resulted in increased IFN-γ in comparison with sham-immunized controls (chapter 5). Nevertheless, and despite the fact that a successful parasite-specific immune response was induced in immunized calves, we were at this time unable to determine the host protective effect of this protocol. Although this is a point certainly worth assessing in future studies, cattle oral based infection with *N. caninum* could prove a significant challenge to overcome. In this regard, oral infection in cattle has only been achieved using *N. caninum* oocysts isolated from experimentally infected dogs[64,65]. Furthermore, so far no viable *in vitro* protocol has been developed for the production of either oocysts or tissue cysts of *N. caninum*.

Altogether these results indicate that our i.n. immunization protocol is capable of inducing a parasite-specific humoral and cell-mediated immune response in cattle. More importantly, a parasite-specific Th1 immune response

was induced in immunized calves, which is a good indicator that this protocol could be host protective against *N. caninum* challenge.

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Chapter 8

Final discussion

8.1 Final Discussion

Concluding remarks and future perspectives

Neospora caninum is a parasite transmitted vertically with high efficiency in cattle[1,2] and responsible for increased abortion rates leading to high economic losses worldwide[3]. The lack of effective strategies to control this protozoan transmission[4,5] and infection has been the driving force behind the majority of the studies performed with this parasite which have greatly contributed to the understating of the host-parasite relationship during infection[6-9]. Although the life cycle of *N. caninum*, decoded for over a decade[1,10] following the identification of the parasite's definitive host[11], and despite the fact that the intestinal mucosa has been established as the crucial site for the initiation of infection in horizontally transmitted neosporosis, the understanding of the host-parasite interactions at this mucosa has been largely overlooked.

In chapter 3 and taking into consideration the first proposed objective of this thesis, a previously developed murine model of i.g. established neosporosis[12] was used to study the immune response elicited following parasite infection. We have focused this work on the elicited cell-mediated immune response due to its well established importance for host resistance against this parasite[13,14]. Nevertheless, previous work by our group has already shown that a parasite-specific humoral immune response, both systemic and in the intestinal mucosa, is also elicited following i.g. infection with *N. caninum*[12]. As depicted in the schematic model summarizing our findings represented in Figure 8-1, it was found that an i.g. challenge with *N. caninum* rapidly stimulates the production of IL-12 by different DC populations in the MLN. In accordance with this result, an early increase in IFN-γ production was observed in $CD8\alpha\beta^{+}TCR\alpha\beta^{+}$ IEL on the intestinal mucosa as well as in $CD8^{+}$ T cells in both the spleen and MLN, while only on later time-points $CD4^{+}$ T cells in the spleen were shown to be activated. Interestingly, although $CD4^{+}$ T cells are thought to have a more prominent role than $CD8^{+}$ T cells in host resistance against neosporosis[15], these results indicate that $CD8^{+}$ T cells could be a key population in the early control of parasite dissemination following i.g. infection.

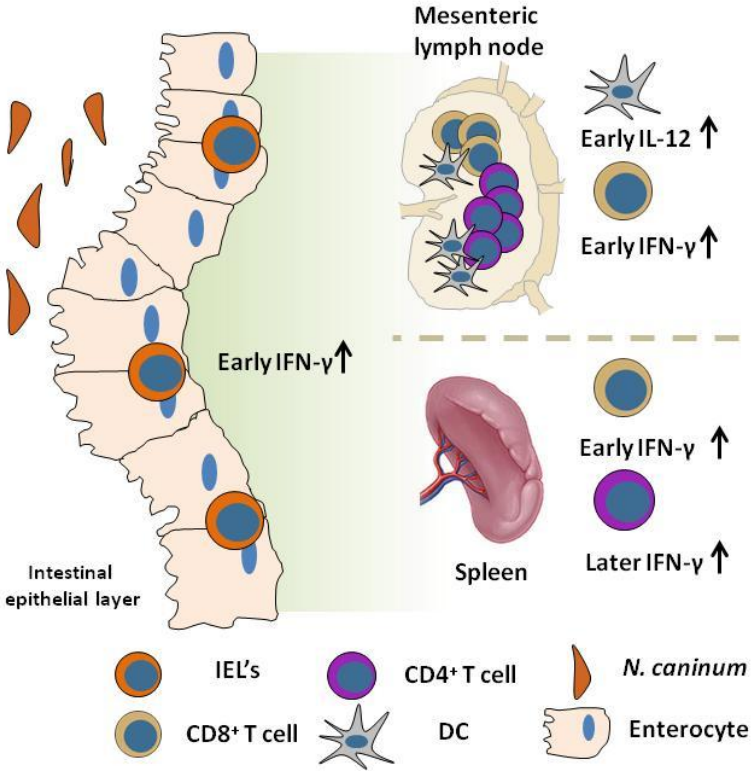

Figure 8-1 – Schematic representation of the results shown in chapter 3

In fact, a very recent study by our group involving i.p. infection of $CD8^{-/-}$ mice confirmed that mice without this cell population were also resistant to *N. caninum* infection but nevertheless presented increased parasitic burdens in different organs which shows that $CD8^{+}$ T cells are important in the early control of parasite proliferation[16]. In this regard, it would be worth assessing in future studies if $CD8^{+}$ T cells in the intestinal mucosa do indeed have a role in limiting parasite dissemination through the host. Additionally, results presented here show that in the first days after i.g. infection the host initiates a Th1-type immune response, in the intestinal mucosa and associated lymphoid tissue, deemed essential for host protection against this parasitic infection[17-20]. Although similar results were observed in mice infected with the closely related protozoan *T. gondii,* where $CD8^{+}$ T cells were also shown to be early responders to parasitic challenge[21], this is the first time that a more in depth characterization of the host immune response in the intestinal mucosa following *N. caninum* infection has been performed.

The second objective of this thesis was to develop and establish a mucosal immunization protocol capable of conferring protection against i.g. established neosporosis. Due to the importance of the intestinal mucosa in the establishment of *N. caninum* infection it was hypothesized that the induction of a parasite-specific immune response in the intestine trough mucosal immunization could be a privileged strategy to confer host protection against neosporosis. Previous studies with *T. gondii* have already demonstrated that following i.n. immunization a parasite-specific mucosal immune response conferred protection against oral infection with this parasite[22,23]. Although i.n. immunization against *N. caninum* has already been performed in mice[24-26], evaluation of the conferred protection was made through i.p. infection, thus overlooking and neglecting the possible host protective effect of mucosal immunity.

In chapter 4 of this thesis we have confirmed our proposed hypothesis and successfully established a host protective i.n. immunization protocol using *N. caninum* membrane proteins and CpG adjuvant in a mouse model of i.g. established neosporosis. As represented in figure 8-2, this protection was accompanied by the production of parasite-agglutinating intestinal IgA and serum parasite-opsonizing IgG. Somewhat unexpectedly considering the IgG isotype analysis and the known host protective role of IFN-γ against this parasitic infection[17-19], immunized mice did not presented increase production of this cytokine. Although a reduction in *N. caninum* colonization levels through immunization has been shown to be possible in the absence of a strong Th1-type immune response[24-28], the immune mechanisms mediating such protection were not yet characterized. Nevertheless, since antibodies specific for membrane proteins of *N. caninum* have been shown to prevent parasite entry into host cells[29-32], we hypothesized that the described effector functions of parasite-specific antibodies elicited by the immunization protocol assessed here, could mediate the observed protection. That would explain the absence of detectable parasite colonization observed in the majority of immunized mice, by immune exclusion of the parasite in the intestinal mucosa due to parasite-agglutinating IgA and parasite-opsonizing IgG. As such and although a biased Th-1 type immune response could have been induced in immunized mice, the absence of increase

IFN-γ production could be explained by a lack of antigenic stimulation of the host immune response as a consequence of the antibody-mediated protection.

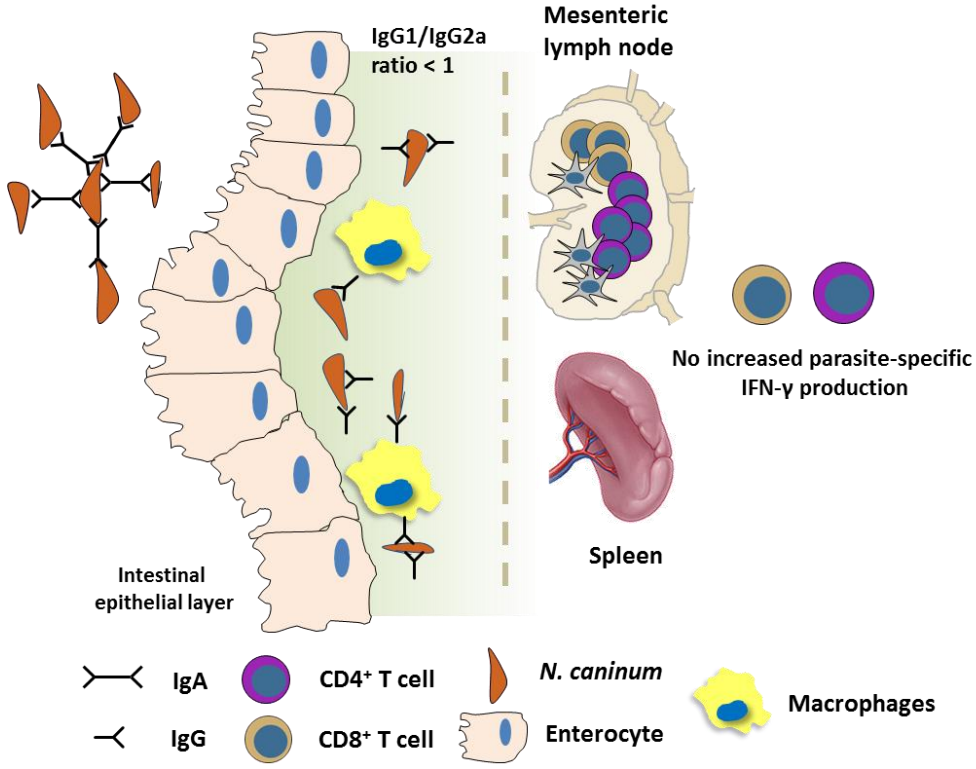

Figure 8-2 – Schematic representation of the results and proposed model of protection in chapter 4

Following the results and model proposed in chapter 4, in chapter 5 the long-term protective effect of the immunization procedure used here was studied. Although an essential characteristic for a successful vaccine, the long-term protective effect of any immunization strategy against neosporosis has never been assessed before. Here, it was demonstrated that protection against i.g. established neosporosis conferred by i.n. immunization with NcMP and CpG was long-lasting. Importantly, the maintenance at long-term of a parasite-specific IgA response in the intestinal mucosa of immunized mice was demonstrated. Although antigen-specific IgA production at this mucosa has been described as decreasing overtime in the absence of antigenic stimulation[33], our data confirms other reports[34] showing that a long-lasting production of antigen-specific IgA can be achieved through mucosal immunization in the intestine. Cytokine production analysis confirmed that induction of a Th1-type immune response was indeed

achieved in immunized mice, since parasite-specific stimulation of MLN or spleen cells resulted in increased IFN-γ production. This result also confirms the presence of parasite-specific cells in MLN nodes following i.n. immunization thus showing that activated cells in the nasal mucosa can migrate and possibly acquire a resident-memory phenotype in lymphoid organs associated to the intestinal mucosa. However further studies will be necessary to fully characterize which cell types were responsible for this increased IFN-γ production, was well as their possible memory phenotype. Still, and in accordance with the results obtained in chapter 4, similar analysis performed one week following i.g. infection once again revealed no significant differences in IFN-γ production between immunized and sham-immunized controls. Furthermore, when comparing pre- and post-infection IFN-γ production in chapter 5 these were found to be very similar in immunized mice and thus in agreement with the proposed model of protection, through prevention of parasite penetration into the host and consequent lack of antigenic stimulation. Nonetheless, and taking into account the results in chapter 3, in chapters 4 and 5 the existence of a parasite-specific cellular immune response in the intestinal mucosa effector sites was not demonstrated. The possibility that the observed protection could also be mediated by parasite-specific IFN-γ producing cells directly in the intestinal mucosa cannot therefore be ruled out. This point needs to be addressed in future studies. Interestingly, in mice immunized i.n. against *T. gondii*, IEL were shown to mediate host protection against toxoplasmosis[35].

In chapter 6 and in order to confirm if host protection conferred by the i.n. immunization could indeed be achieved in the absence of a Th1-type immune response, we assessed it in a highly susceptible IL-12-defiecient mice[36]. Once again, and confirming the model proposed here, host protection characterized by lack of detectable parasitic DNA could still be observed in i.g infected immunized $p40^{-/-}$ mice. Nevertheless, when parasitic DNA was detected in immunized mice it reached levels similar to those observed in sham-immunized controls. This result further supported the hypothesis that prevention of host infection could be due to mucosally produced IgA. However and due to the marked susceptibility of IL-12-deficient mice to *N. caninum* infection[36], if the parasite could overcome this initial mucosal protective mechanism, than it could be expected that this could easily

disseminate in the host due to a compromised Th-1 cell immunity. As such and in order to confirm the host protective effect of parasite-specific intestinal IgA, similarly immunized p40$^{-/-}$ mice were i.p. infected allowing the parasite to overcome the intestinal barrier. However and surprisingly, immunized mice were still protected following *N. caninum* i.p. challenge. Furthermore, some mice even had undetectable parasitic DNA. Since previous studies in IL-12-deficient mice have shown that an increase IFN-γ production can be induced trough immunization prior to infection[37] it can be hypothesized that a similar mechanism could account for the observed protection since a smaller but still below 1 IgG1/IgG2a ratio was observed in immunized p40$^{-/-}$ mice. Nevertheless the analysis of IFN-γ production by splenocytes from mice infected i.p. did not revealed such an increase. This could be due to different kinetics of IFN-γ production between sham and immunized mice. To assess this, analysis done prior to and in different time points after i.p. infection would help to clarify this point. Nonetheless, and taking into consideration the protection profile observed in i.g. infected p40$^{-/-}$ mice, and if a higher production of IFN-γ could have been induced in the immunized mice it is intriguing as to why a similar reduction in parasitic burden was not observed in i.g. infected immunized p40$^{-/-}$ mice. The stimulation of different immune cell populations in the different infection routes could be a contributing factor for these discrepancies. For example, and due to the known role of DC in the spreading of the parasite trough the host[38], the early encounter of activated DC in the MLN of mice i.g. infected as described in chapter 3 could indicate that if the infection is established through the intestinal mucosa this population could be responsible for the trafficking and shielding of the parasite from the host immune response. This is a point certainly worth exploring in future studies. In addition, and taking into account the widespread use of i.p. infection for the evaluation of the host protective effect of nearly all immunization studies in murine neosporosis to date[7,9], these results indicate that different infection routes could lead to different outcomes in terms of protection. As such, i.g. infection route more closely represents the route followed by the parasite in horizontally transmitted neosporosis. On the other hand, the host protective effect observed in i.p. infected immunized p40$^{-/-}$ mice did not allow to conclude that the mucosal barriers, such as intestinal IgA, are indeed responsible for the protection observed in i.g. infected immunized mice. In this regard, the use of mice deficient on IgA

production[39] or its translocation to the intestinal mucosa[40] could allow a more definitive conclusion on this matter in future studies. Nevertheless, and taking into consideration the initially stated objectives of this thesis, a novel mucosal immunization protocol capable of conferring long-lasting protection against i.g. established neosporosis was achieved and it could also be effective in mice presenting a compromised cell-mediated immunity.

Lastly, in chapter 7 the final proposed objective was addressed and it was confirmed that the used i.n. immunization could elicit a parasite-specific humoral and cellular immune response in cattle. Although limited in scope, this initial analysis confirmed the feasibility and immunogenicity of this immunization strategy in the final target species, the bovine. Furthermore, for the first time a mucosal immunization protocol was used in cattle as a means to confer host protection against *N. caninum,* which opens new perspectives for future alternatives to achieve control of this parasitosis. The analysis of host protection in immunized cattle will be an essential point to be addressed in future studies, although the establishment of an oral based infection in cattle will be a challenging proposition.

Altogether, the results presented in this thesis further expanded the knowledge on the host immune response in the intestinal mucosa of *N. caninum* infected mice. This knowledge may help in the rational development of novel strategies to control this parasitic disease. In addition, the developed mucosal immunization protocol presented here was capable of inducing mucosal and systemic parasite-specific immunity that could confer long-lasting protection against *N. caninum* infection.

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Annex 1

Mucosal and systemic T cell response in mice intragastrically infected with *Neospora caninum* tachyzoites

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Mucosal and systemic T cell response in mice intragastrically infected with *Neospora caninum* tachyzoites

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Abstract

The murine model has been widely used to study the host immune response to *Neospora caninum*. However, in most studies, the intraperitoneal route was preferentially used to establish infection. Here, C57BL/6 mice were infected with *N. caninum* tachyzoites by the intragastric route, as it more closely resembles the natural route of infection through the gastrointestinal tract. The elicited T-cell mediated immune response was evaluated in the intestinal epithelium and mesenteric lymph nodes (MLN). Early upon the parasitic challenge, IL-12 production by conventional and plasmacytoid dendritic cells was increased in MLN. Accordingly, increased proportions and numbers of $TCR\alpha\beta^+CD8^+IFN\text{-}\gamma^+$ lymphocytes were detected, not only in the intestinal epithelium and MLN, but also in the spleen of the infected mice. In this organ, IFN-γ-producing $TCR\alpha\beta^+CD4^+$ T cells were also found to increase in the infected mice, however later than $CD8^+$ T cells. Interestingly, splenic and MLN $CD4^+CD25^+$ T cells sorted from infected mice presented a suppressive activity on in vitro T cell proliferation and cytokine production above that of control counterparts. These results altogether indicate that, by producing IFN-γ, $TCR\alpha\beta^+CD8^+$ cells contribute for local and systemic host protection in the earliest days upon infection established through the gastrointestinal tract. Nevertheless, they also provide substantial evidence for a parasite-driven reinforcement of T regulatory cell function which may contribute for parasite persistence in the host and might represent an additional barrier to overcome towards effective vaccination.

Introduction

Neospora caninum is a protozoan parasite found in a wide range of domestic and wild animal hosts [1], and is responsible for clinical infections in dogs and cattle [2], having a major impact in dairy and beef industry [3]. Experimentally, the murine model has been the one preferred to study neosporosis, as it presented similar features to the infection occurring naturally in permissive hosts such as brain lesions [4], reproductive loss [5] and mother to fetus parasite transmission [6]. Although *N. caninum* is transplacentally transmitted in cattle with high efficiency, significant postnatal transmission also occurs in these animals [1], likely through oocyst ingestion [7]. Even though neosporosis can thus be established through the gastrointestinal (GI) tract, most studies on the host immune response have been carried out in hosts infected via the intraperitoneal (i.p.) or subcutaneous routes. Consequently, the mucosal immune response to this parasite in infected hosts was barely studied. As mucosal immunizations have been already attempted in experimental models of neosporosis [8-10], the characterization of the immune response to *N. caninum* in the mucosa and associated lymphoid tissues will be helpful to further understand the immunobiology of this parasitic disease. Therefore, a murine model of neosporosis established by intragastric (i.g.) administration of *N. caninum* tachyzoites was used here to study the immune response elicited by this parasite in the gut and associated lymphoid tissue of the infected hosts.

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Materials and methods

Mice

Female C57BL/6 mice, 8–10 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific pathogen-free conditions at the Animal Facility of Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Porto, Portugal. Female $p40^{-/-}$ C57BL/6 mice, 7–11 weeks old, were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and housed and bred also at ICBAS in individual ventilated cages. Nesting and housing material was provided as enrichment. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123), 86/609/EEC Directive and Portuguese rules (DL 129/92). Authorization to perform the experiments was issued by the competent national board authority, Direcção Geral de Veterinária (0420/000/000/2008).

Parasites

Neospora caninum tachyzoites (NC-1 isolate) were cultured and serially passaged in VERO cells maintained at 37 °C in Minimum Essential Medium (MEM) containing Earle's salts (Gibco: Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (200 IU/mL) and streptomycin (200 μg/mL) (all from Sigma, St Louis, USA) in a humidified atmosphere of 5% CO_2 in air. Free parasitic forms of *N. caninum* were obtained as previously described [11] with slight modifications. Infected VERO cells were cultured until the host cell monolayer was 90% destroyed. Culture supernatants and adherent cells, harvested using a cell scraper, were centrifuged at $1500 \times g$ for 15 min. The pellet was passed through a 25G needle and then washed three times in Phosphate Buffered Saline (PBS). The obtained pellet was suspended in 3 mL of PBS and passed through a PD-10 column filled with Sephadex™ G-25 M (Amersham Biosciences Europe GmbH, Freiburg, Germany). Parasite concentration was determined with a haemocytometer.

Challenge infections

N. caninum infections in C57BL/6 mice were performed by the i.g. route using a previously described protocol [11]. Briefly, 5 h before infection mice were deprived of food. Mice were then anaesthetized by intramuscular injection of 20 μL of a 4:5 mixture containing xylazine (Rompum®, Bayer Portugal, S.A., Carnaxide) and ketamine (Imalgéne 1000, Bayer Portugal, S.A., Carnaxide). Stomach acidity was neutralized by directly administering into the stomach, with a gavage feeding needle linked to a 1-mL syringe, 50 μL of a 10% sodium bicarbonate solution in water. The same procedure was used to inoculate *N. caninum* tachyzoites 15 min later. Mice were i.g. challenged with 5×10^7 tachyzoites in 0.2 mL of PBS or similarly inoculated with 0.2 mL of PBS and sacrificed at 6 h, 12 h, 18 h, 48 h, and 4, 7 and 21 days after challenge.

Sample collection

At the different time points, mice were sacrificed upon isoflurane anesthesia by cervical dislocation. Spleens and mesenteric lymph nodes (MLN), from infected mice and non-infected controls, were aseptically removed and homogenized to single cell suspensions in HBSS for their usage in cell culture experiments and flow cytometry analysis. Additionally, brain, liver, MLN, and intestinal tissue samples were collected and either frozen (DNA isolation) or formalin-fixed (histology and immunohistochemistry). Small intestines were alternatively collected for IEL isolation. The number of animals per group per experiment is indicated in the respective figure legends.

Histopathology and immunohistochemistry

Histopathology of intestinal tissue samples was assessed in formalin-fixed, paraffin-embedded 4 μm sections, mounted on amino-propyl-tri-ethoxy-silane (Sigma-Aldrich, St Louis, MO, USA) coated slides, of the gastrointestinal tract of mice 6, 12 and 18 h upon i.g. infection, stained with haematoxylin-eosin. The presence of *N. caninum* parasitic forms was assessed in similar tissue sections by immunohistochemistry, performed as previously described with slight changes [11]. Tissue sections were deparaffinized in xylene, rehydrated by graded washes of ethanol in water, ending in a final rinse in deionized water. Antigen retrieval was performed by incubating the slides in 10 mM citrate buffer (pH = 6) for 3 min in a pressure cooker. The slides were cooled and rinsed three times in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH = 7.6) for 5 min. Endogenous peroxidase activity was blocked by immersing slides in methanol containing 3% hydrogen peroxide for 10 min, followed by TBS washing. To reduce non-specific antibody binding, slides were incubated with normal rabbit serum (Dako, Glostrup, Denmark) diluted at 1:5 in TBS containing 10% bovine serum albumin (BSA), in a humidified chamber for 20 min at room temperature. Excess normal serum was removed and replaced by the goat anti-*N. caninum* antiserum (VMRD, Pullman, WA, USA) diluted at 1:2000. After one hour incubation at room temperature, slides were washed with TBS and incubated for 30 min with a 1:1000 dilution of peroxidase-labeled rabbit anti-goat secondary antibody (Millipore, Billerica, MA, USA). Slides were then washed with TBS and detection was performed for 3 to 5 min with 0.05% 3,3 diaminobenzidinetetrahydrochloride (DAB) freshly prepared in

0.05 M Tris/hydroxymethylaminomethane buffer, pH 7.6, containing 0.1% hydrogen peroxide (Dako). Finally, sections were lightly counterstained with Mayer's haematoxylin, dehydrated and mounted in Entellan® mounting medium (Merck, Darmstadt, Germany). Dilution of primary antibody and peroxidase-labeled secondary antibody were made with TBS containing 5% BSA. Positive control sections of *N. caninum*-infected IL-12$^{-/-}$ mouse organs were included. Negative controls were performed by omitting the primary antibody incubation. Slides were evaluated under light microscopy.

Real-time PCR analysis

DNA from intestinal tissue sections, MLN, liver and brain was isolated as previously described [11]. Detection of *N. caninum* DNA in infected tissue samples was assessed by a quantitative real-time PCR (qRT-PCR) analysis performed in a Corbett rotor gene 6000 system (Corbett life science, Sydney, Australia), using Express Sybr green ER qPCR supermix universal (Invitrogen, Carlsbad, CA, USA), for the amplification of a 337 bp sequence of the Nc5 region of *N. caninum* genome using the primers Np21plus 5' CCCAGTGCGTCCAATCCTGTAAC 3' and Np6plus 5' CTCGCCAGTCAACCTACGTCTTCT 3' (TIB-Molbiol, Berlin, Germany), both at a final concentration of 0.5 μM. The DNA samples were amplified using the following program: 95 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, 63 °C for 20 s, and 72 °C for 45 s with fluorescence acquisition. A melting curve was performed in each run in order to confirm specificity of the amplicon: from 65 °C to 95 °C, with increments of 1 °C for 5 s. Parasite quantification was determined by interpolation of a standard curve, ranging from 10 to 10^{-4} ng of DNA extracted from *N. caninum* tachyzoites included in each run. Data were analyzed in the Rotor gene 6000 software v1.7 (Corbett life science).

Intraepithelial lymphocyte isolation

Gut intraepithelial lymphocytes (IELs) were isolated as previously described [12]. Briefly, mice small intestines were removed and flushed with 20 mL of cold CMF (Ca^{++}, Mg^{++} free Hank's Balanced Salt Solution (HBSS) with 1 mM HEPES and 2% FBS, all from Sigma) using a syringe and needle. The Peyer's Patches, fat and remaining mucous were removed along the intestine. The intestine was opened lengthwise, cut into 5 mm pieces and placed in a conical tube with 40 mL CMF. The pieces of tissue were washed twice with CMF by inverting the tube 10 times and letting the pieces settle before removing the supernatant. The intestine pieces were incubated in 25 mL CMF/DTE (CMF with 10% FBS and 1 mM dithioerythritol) (Sigma) at 37 °C and 100 rpm in an orbital incubator (GFL 3031, GFL, Burgwedel, Germany) for 20 min. The tube was vortexed at maximum speed for 15 s and the supernatant removed to a new tube. 25 mL of CMF/DTE were added to the tube containing the pieces of tissue and the vortexing step and collection of supernatant were done once more. All the incubation and supernatant collection steps were repeated. Supernatants from each intestine were pooled and centrifuged at 400 × *g*, 4 °C for 20 min. The pellet was suspended in 5 mL HBSS with 2% FBS and passed through a nylon wool column pre-wet with HBSS with 2% FBS (0.15 g teased nylon wool in a 5 cc syringe) and the column was washed with 20 mL HBSS with 2% FBS. The collected cell suspension was centrifuged and suspended in 16 mL of 44% Percoll™ (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), adding 8 mL per 14 mL polystyrene round bottom tube. 5 mL of 67% Percoll were underlaid per tube and the 44%/67% Percoll gradients were centrifuged for 20 min at room temperature, 1400 × *g*, with the brake off. Cells from the interface were carefully removed with a Pasteur pipette and washed twice with 40 mL cold complete RPMI (RPMI 1640 supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, 1% HEPES buffer, 10% FCS and 5 μM 2-mercaptoethanol, all from Sigma). Pellet was suspended in complete RPMI.

Flow cytometric analysis

The assessment of cell surface and cytoplasmic lineage or activation markers on different splenic leukocyte populations was performed by flow cytometric analysis (FACS). From spleen and MLN cell suspensions, prepared as described above, a number of 1 × 10^6 leucocytes were stained per sample. The following monoclonal antibodies (mAbs), along with the respective isotype controls were used (at previously determined optimal dilutions) for immunofluorescence cytometric data acquisition in a Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., Brea, CA, USA): fluorescein isothiocyanate (FITC) anti-mouse/rat Foxp3 (FJK-16 s), phycoerythrin (PE) anti-mouse TCR β (H57-597) and PE-Cy5 rat anti-mouse CD4 (L3T4) (RM4-5) (all from eBioscience, San Diego, CA, USA); Biotin anti-mouse PDCA-1 (JF05-1C2.4.1) (Miltenyi Biotech, Inc. Auburn, CA, USA); FITC hamster anti-mouse CD11c (HL3), PE-Cy5 rat anti-mouse CD8a (53–6.7), PE anti-mouse CD25 (PC61), PE rat anti-mouse IL-4 (BVD4-1D11), Biotin hamster anti-mouse γδ T-cell receptor (GL3), FITC anti-mouse IFN-γ (XMG1.2), FITC rat anti-mouse IL-17A (TC11-18H10), PE rat anti-mouse IL-10 (JES5-2A5) (all from BD Pharmingen, San Diego, CA, USA). Biotin conjugated mAbs were revealed with Streptavidin-PE-Cy7 (BD Pharmingen). Cells were preincubated for 15 min with anti-FcγR (a kind gift of

Dr Jocelyne Demengeot, Gulbenkian Institute of Science, Oeiras, Portugal) before CD11c and Foxp3 staining. The Foxp3 Staining Buffer Set (eBioscience) was used for fixation and permeabilization of splenocytes previously surface stained with CD4 and CD25 mAbs. Data were analysed by using CELLQUEST software (Becton-Dickinson, San Jose, CA, USA).

Intracellular staining

The intracellular expression of the cytokine IFN-γ was detected in splenic and MLN $CD8^+$ and $CD4^+$ T lymphocytes, as well as in IELs. IL-4, IL-17A and IL-10 expression was also evaluated in splenic and MLN $CD4^+$T cells. Splenocytes and MLN cells were obtained as described above. Red blood cell lysis was performed in spleen suspensions by incubation with 0.15 M ammonium chloride. Cells were washed and suspended in complete RPMI medium. Spleen, MLN or IEL 1×10^6 cells were transferred to 96-well tissue culture plates (Nunc, Roskilde, Denmark) and stimulated for 4.5 h with 20 ng/mL phorbol myristate acetate and 200 ng/mL ionomycin in the presence of 10 ng/mL of brefeldin A (all from Sigma). Staining of cell surface markers CD4, CD8, TCR β and TCRγδ was performed as described above, after a preincubation step of 15 min with anti-FcγR, followed by fixation with 2% formaldehyde. Cells were permeabilized with 0.5% saponin in flow cytometric buffer (PBS containing 1% BSA and 0.01 M sodium azide) and, subsequently, cells were incubated for 15 min with anti-FcγR and stained for 30 min at room temperature with the appropriate antibody. The intracellular expression of the cytokines IL-12 and IL-10 was detected in splenic and MLN conventional and plasmacytoid dendritic cells (cDC and pDC, respectively). Spleen and MLN suspensions were enriched with DC by magnetic sorting using anti-CD11c beads (Miltenyi), according to the manufacturer's instructions. 1×10^6 cells were then placed in 96-well tissue culture plates (Nunc) and incubated for 4.5 h with 10 ng/mL of brefeldin A (Sigma). Cells were surface stained with PE-Cy5.5 hamster anti-mouse CD11c (HL3) (BD Pharmingen) and Biotin anti-mouse PDCA-1 (Miltenyi Biotech) revealed with PE-Cy7 conjugated streptavidin (BD Pharmingen). Intracellular staining was performed with PE rat anti-mouse IL-12 (p40/p70) (C15.6) and FITC rat anti-mouse IL-10 (JES5-2A5) (all from BD Pharmingen). Intracellular staining with the isotypic controls was performed to confirm the specificity of antibody binding.

Cell cultures and suppression assays

For anti-CD3 mAb-stimulated cultures, antigen presenting cells (APC) were prepared from naïve splenic or MLN single cell suspensions by layering 5 mL onto 2.5 mL of a polysucrose-sodium ditrizoate solution (Histopaque 1083®, Sigma) and centrifuging at 800 × *g* for 20 min at room temperature. Mononuclear cells collected from the medium-Histopaque interface were washed, suspended in RPMI complete medium and irradiated at 3000 rad in a Gammacell 1000 Elite irradiator (Nordion International, Inc., Ottawa, Canada). Total $CD4^+$ and the T cell subsets $CD4^+CD25^-$ and $CD4^+CD25^+$ from non-infected and infected mice were isolated from pooled spleen or MLN cells of five mice per group, by using a magnetic cell sorting $CD4^+CD25^+$ T-cell isolation kit (Miltenyi Biotech, Inc., Auburn, CA, USA) following the manufacturer's instructions. Purity of the sorted cells routinely ranged between 92-98%. Naïve $CD4^+CD25^-$ T cells (responder cells) were plated at 2.5×10^4/well in U-shape 96-well plates together with 10^5 APC without stimulus or stimulated with 1 μg/mL anti-CD3 mAb (145.2C11) (BD Pharmingen). $CD4^+CD25^+$ T cells sorted from control and *N. caninum*-infected mice were added to the naïve $CD4^+CD25^-$ T cells in a 1:1 proportion. Each condition was set in sextuplicates and culture was maintained for 72 h. Supernatants from these cell cultures were collected and stored at −80 °C until further use. The CellTraceTM CFSE Cell Proliferation Kit (Molecular Probes, Invitrogen, Eugene, OR, USA) was used for cell labelling. A CFSE (5-(and-6)-carboxyfluorescein diacetate succinimidyl ester) stock solution (10 mM in DMSO) stored at −20 °C was thawed and diluted in PBS with 0.1% BSA to a final concentration of 10 μM. Naïve $CD4^+CD25^-$ T cells (responder cells) were suspended at 2×10^6/mL in PBS with 0.1% BSA and further incubated with an equal volume of the diluted CFSE solution, for 7 min at room temperature. Excess CFSE was quenched by adding 1/5 of the volume of heat inactivated FBS. Cells were washed three times with complete RPMI medium. Responder cells were plated at 2.5×10^4/well in U-shape 96-well plates together with 10^5 APC and 1 μg/mL anti-CD3 mAb. In order to evaluate Treg cell suppressive function, $CD4^+CD25^+$ T cells from control and infected mice were added at different responder: $CD4^+CD25^+$ T cell ratios (1:1, 2:1, and 10:1). Responder cells without anti-CD3 stimulus were used as the negative control. Stimulated responder cells with no suppressor populations added were used as the positive control. Unlabelled stimulated responder cells were used to define cell auto fluorescence. Other controls consisted of stimulated responder cells co-cultured with $CD4^+CD25^-$ T cells from the different animal groups tested, to exclude suppression due to cell number/well. Each condition was set in sextuplicates and cultures were maintained for 72 h at 37 °C and 5% CO_2. Proliferation/suppression was determined based on CFSE fluorescence by flow cytometric analysis.

For antigen stimulated cultures, bone marrow-derived dendritic cells (BMDC) were prepared by a granulocyte macrophage colony-stimulating factor (GM-CSF)-based method, as described by Lutz et al. [13]. Upon differentiation, BMDC were antigen-loaded by overnight incubation with 100 μg/mL of *N. caninum* sonicates prepared as previously described [11], or cultured without antigen in the presence of 50 ng/mL of lipopolysaccharide from *E. coli* (Sigma). BMDC APC were then washed twice with PBS and suspended in complete RPMI. CFSE-labelled responder cells ($CD4^+CD25^-$ cells isolated, as described above, from infected mice, 7 days upon infection) were plated at 2.5×10^4/well in U-shape 96-well plates together with 10^5 BMDC and were used as the positive control. To evaluate Treg suppression capacity, $CD4^+CD25^+$ T cells from control and infected mice were added to the cultures at 1:1 and 2:1 responder: $CD4^+CD25^+$ T cell ratios.

IFN-γ, IL-4, and IL-10 measurements

The concentration of IFN-γ, IL-4 and IL-10 in cell culture supernatants from *N. caninum*-infected mice and from non-infected controls were quantified with the Mouse IFN-γ, IL-4 and IL-10 ELISA Ready-Set-Go!® kits (eBioscience), according to manufacturer's instructions.

Statistical analysis

Unless otherwise indicated, statistical significance of results was determined by unpaired Student *t*-test, using the GraphPad Prism 4 Software (GraphPad Software, Inc., La Jolla, CA, USA). Results were considered statistically significant with P values of less than 0.05.

Results

Infection of C57BL/6 mice with *N. caninum* tachyzoites administered intragastrically

We have previously reported that neosporosis could be established in mice i.g. challenged with *N. caninum* tachyzoites. However, in the i.g.-challenged mice, the immune response elicited in the gut associated lymphoid tissue was studied only in the Peyer's Patches [11]. Here, C57BL/6 mice were challenged i.g. with *N. caninum* tachyzoites to evaluate the elicited immune response in the gut epithelium and draining lymph nodes. Early after the i.g. challenge, parasitic DNA was detected by qRT-PCR in intestinal tissue samples (1/4, 2/4 and 1/4 mice at 6, 12 and 18 h, respectively) and MLN (1/4 mice at 6 h). The presence of tachyzoites within the intestinal tissue was confirmed by immunohistochemistry (Additional file 1). No evident signs of inflammation were observed in intestinal tissue samples of the infected mice up to 18 h upon infection, as evaluated by microscopic observation of haematoxilin/eosin stained paraffin sections (data not shown). In the infected mice, the presence of *N. caninum* DNA was assessed in the liver and brain at 4 and 7 days upon infection, in pooled samples of two independent experiments. Parasitic DNA was detected by qRT-PCR in both organs in 4/10 mice and 6/10 mice, 4 and 7 days after infection, respectively. Similarly infected C57BL/6 mice still presented parasitic DNA in the brain 21 days after infection (2/4) and 5/5 survived infection for at least six months. A more exhaustive analysis would nevertheless be necessary to determine whether the surviving mice were chronically infected. These results show that in C57BL/6 mice *N. caninum* tachyzoites can disseminate in the host from the GI tract. Results also show that the parasite i.g.-challenged mice control acute infection.

MLN cDC and pDC produce IL-12 early upon i.g. challenge with *N. caninum* tachyzoites

As we have shown in a previous report a large proportion of splenic conventional and plasmacytoid dendritic cells (cDC and pDC, respectively) produce IL-12 in BALB/c mice infected i.p. with *N. caninum* tachy zoites [14]. This cytokine is a crucial factor in mediating host immune protection against neosporosis [15-17]. As shown in Figure 1, an increased frequency of IL-12-expressing cDC and pDC was detected in the MLN of infected mice, 18 and 48 h upon the parasitic challenge. The frequency of IL-10-producing MLN cDC and pDC was also evaluated and did not significantly change upon infection (data not shown). These results show that *N. caninum* tachyzoites administered i.g. induce IL-12 production by host dendritic cells (DC) in the draining lymph nodes.

Increased frequencies of $TCR\beta^+CD8^+IFN\text{-}\gamma^+$ IEL were observed in C57BL/6 mice challenged i.g. with *N. caninum* tachyzoites

Murine gut IEL comprise both αβ and γδ TCR^+ cells [18] which have been shown to mediate host protection against enteric infections, including those caused by protozoans [19]. Here, an increased frequency of $TCR\beta^+$ $CD8^+IFN\text{-}\gamma^+$ IEL was observed in C57BL/6 mice comparatively to mock-infected controls, 48 h upon i.g. challenge with *N. caninum* tachyzoites (Figure 2). Conversely, no differences were found between infected mice and controls in the frequencies of IFN-γ-producing $TCR\gamma\delta^+$ (1,30 ± 0,27 vs 1,13 ± 0,29) or $TCR\beta^+CD4^+$ (2,70 ± 0,82 vs 1,84 ± 0,76) IEL. Production of IL-17A by $TCR\gamma\delta^+$ IEL was not detected either in controls or infected mice. These results indicate that in the gut, $CD8^+\alpha\beta TCR^+$, but not $\gamma\delta TCR^+$, IEL are activated by i.g.-administered *N. caninum* tachyzoites and produce the host protective cytokine IFN-γ.

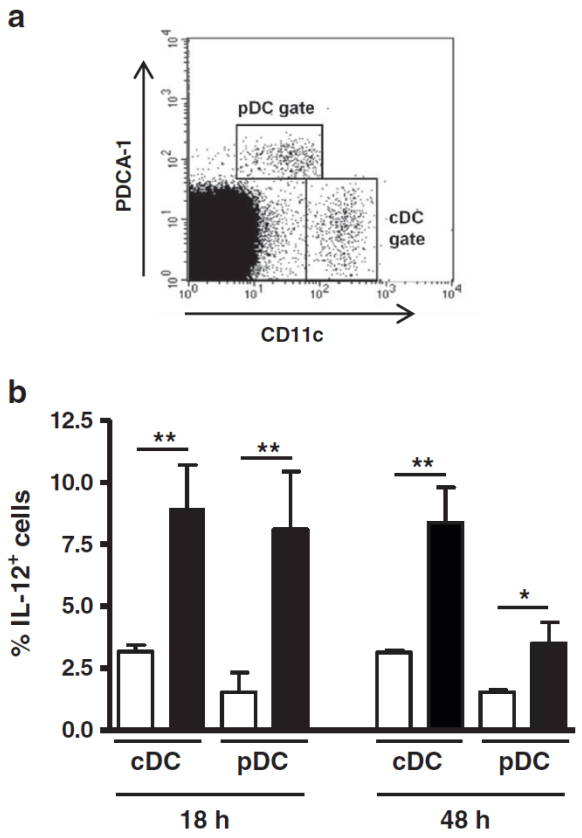

Figure 1 cDC and pDC produce IL-12 in response to *N. caninum* i.g. infection. (a) Representative example of flow cytometric analysis of surface PDCA-1 and CD11c expression on total MLN leukocyte cells. Dot plots represent cells collected from C57BL/6 mice 18 h after i.g. challenge with 5×10^7 *N. caninum* tachyzoites. Gates were set as shown to delineate cDC ($CD11c^{high}$) and pDC ($CD11c^{low}$ $PDCA\text{-}1^{high}$). **(b)** Proportion of $IL\text{-}12^+$ cells within cDC and pDC populations in the MLN of C57BL/6 mice, evaluated by flow cytometric analysis, at the specified time points after i.g. treatment with PBS (open bars) or i.g. inoculation with 5×10^7 *N. caninum* tachyzoites (closed bars). Bars represent the mean plus one standard deviation of three animals in the PBS group and four animals in the infected mice group. This is one representative result of two independent experiments (*P<0.05; **P<0.01).

$CD8^+$ T cells produce IFN-γ in the MLN of *N. caninum* i.g.-infected mice

IL-12 drives the differentiation of T cells towards an IFN-γ-producing phenotype. As increased production of IL-12 was observed in the MLN of *N. caninum* infected mice, the frequency of $CD4^+$ and $CD8^+$ T cells producing IFN-γ was assessed therein, 4 and 7 days upon infection. As shown in Figure 3a, an increased frequency of $CD8^+IFN\text{-}\gamma^+$ T cells was observed in the MLN of infected mice. This increase was observed at day 4 post-infection whereas at day 7 it was found below control values. No such increase was observed for $CD4^+IFN\text{-}\gamma^+$ T cells at the assessed days (Figure 3c). Nevertheless, at day 7 upon infection some mice presented $CD4^+IFN\text{-}\gamma^+$ T cells at higher frequency than controls. This high variability was observed in the three experiments done. In order to determine whether IFN-γ production could also be induced in splenic T cells upon the i.g. infection, their frequency and number was similarly assessed. As shown in Figure 3b, increased proportions of $CD8^+IFN\text{-}\gamma^+$ T cells were also observed in the spleen 4 days upon infection that were found within control values by day 7. In contrast, the frequency of $CD4^+IFN\text{-}\gamma^+$ T cells, which was similar to controls 4 days after infection, significantly increased at day 7 (Figure 3d). The frequency of $CD4^+$ T cells expressing IL-4, IL-10 or IL-17A was not different between controls and infected mice at both assessed time points and lymphoid tissues (data not shown). These results show that in the i.g. infected mice $CD8^+$ T cells are early producers of IFN-γ, not only at the intestinal mucosa and draining lymph nodes but also systemically, as detected in the spleen.

Increased suppressive activity of $CD4^+CD25^+$ T cells from infected mice

We have previously reported that in *N. caninum* i.g.-infected BALB/c mice, higher numbers of $CD4^+$ $CD25^+Foxp3^+$ cells (T regulatory cells, Treg), as well as of $CD4^+CD25^+Foxp3^-$ cells (T effector cells, Teff), were detected in the spleen by 8 days upon the parasitic challenge [11]. Accordingly, increased numbers of $CD4^+$ $CD25^+$ cells were found in the C57BL/6 infected mice at day 7, but not at day 4 upon infection. As *N. caninum* i.g. infection did not significantly change the relative proportions of Treg and Teff (Additional file 2), the numbers of Treg and Teff are proportionally increased (Figure 4). Interestingly, 7 days after the parasitic challenge, the frequency of $CD4^+CD25^-$ cells expressing the Treg marker Foxp3 was increased in the spleen of infected mice (Additional file 2). No such differences were observed 4 days upon infection or at any assessed time point in the MLN (data not shown). To determine whether, regardless of similar Treg and Teff proportions, splenic $CD4^+CD25^+$ T cells sorted from *N. caninum*-infected and control mice could have dissimilar suppressive activity, a suppression assay was performed by co-culturing $CD4^+CD25^+$ T cells sorted from infected or control mice with CFSE-labelled naïve $CD4^+CD25^-$ T cell responders. Interestingly, $CD4^+CD25^+$ T cells obtained from infected mice suppressed more efficiently the anti-CD3 mAb-induced proliferation of T cell responders than did the control $CD4^+CD25^+$ counterparts. This effect was observed at any of the assessed responder: $CD4^+CD25^+$ T cell ratios (1:1; 2:1; 10:1). Curiously, when $CD4^+CD25^-$ T cells from *N. caninum*-infected mice were co-cultured with responder cells, a noticeable suppression of responders proliferation was also observed, which did not occur when $CD4^+CD25^-$ T

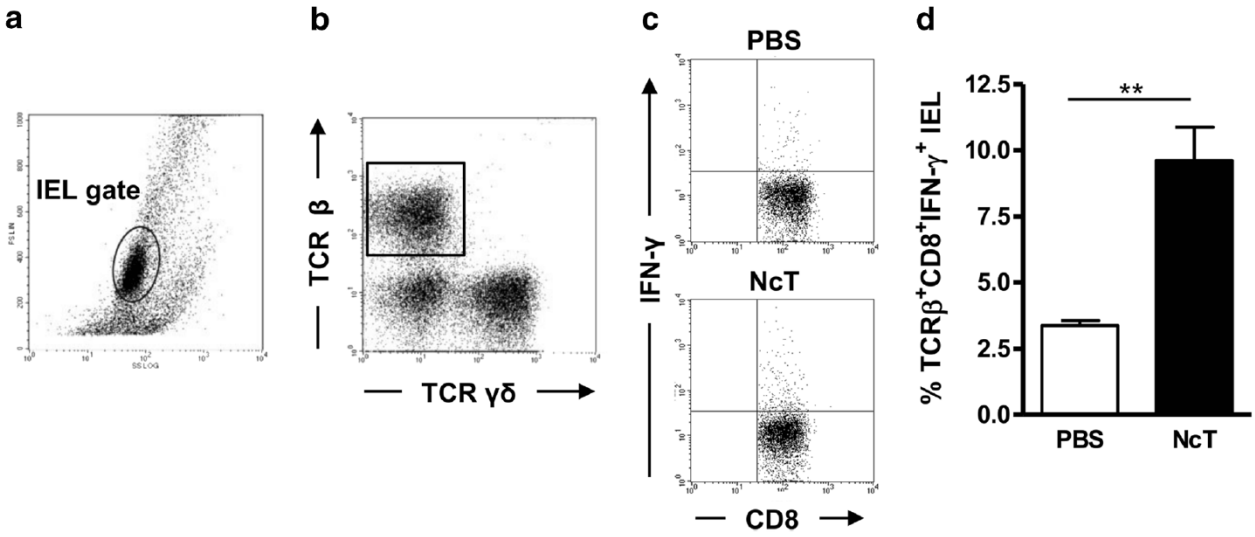

Figure 2 Increased expression of IFN-γ by TCRβ$^+$CD8$^+$ IEL in *N. caninum* infected mice. Representative dot plots of **(a)** cells isolated from the small intestines of C57BL/6 mice (IEL were gated as shown); **(b)** TCRγδ and TCRβ IEL. **(c)** Gated TCRβ CD8$^+$ IEL expressing IFN-γ in control (PBS) and *N. caninum*-infected mice (NcT). **(d)** Frequency of TCRβ$^+$CD8$^+$ IFN-γ$^+$ IEL in control and *N. caninum* i.g.-infected mice, 48 h after challenge. Bars represent mean plus one standard deviation of three animals in the PBS group and four animals in the infected mice group. This is one representative result of three independent experiments (*P<0.05).

cells from controls were used (Figure 5a). This might be a consequence of the increased frequency of Foxp3-expressing cells within this cell subset detected in the 7-day infected mice, which in mice are known to have a regulatory function [20]. In order to determine whether the observed disparate suppressive effect of Treg from infected and control mice could also occur in *N. caninum*-antigen stimulated cultures, antigen-loaded BMDC were used as APC to stimulate T cell proliferation/suppression. Activation of BMDC upon incubation with *N.*

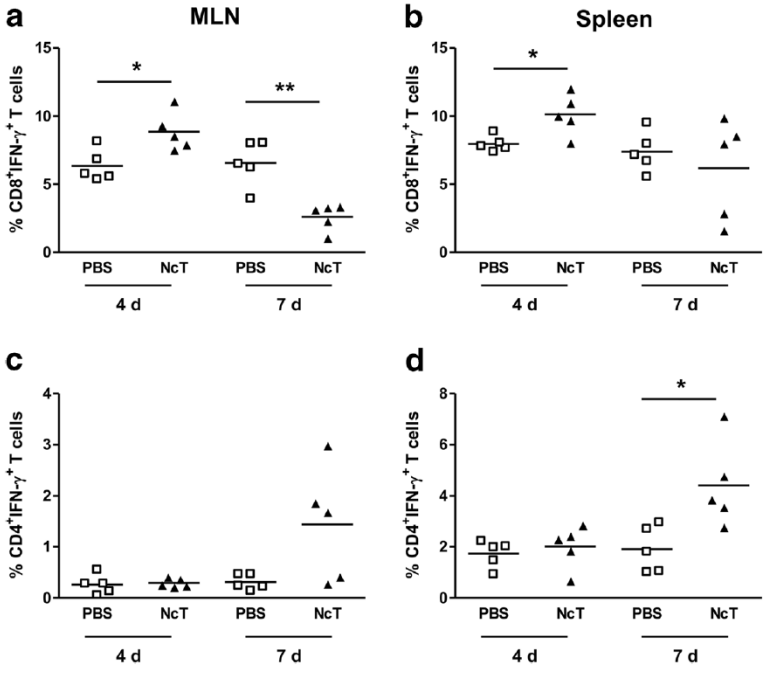

Figure 3 Expression of IFN-γ in splenic and MLN CD8$^+$ and CD4$^+$ T cells. Scatter plots of frequency of CD8$^+$IFN-γ$^+$ T cells **(a** and **b)** or CD4$^+$IFN-γ$^+$ T cells **(c** and **d)**, as indicated, in the MNL **(a** and **c)** and spleen **(b** and **d)** of control (PBS) or *N. caninum*-infected mice (NcT), 4 and 7 days upon i.g. challenge. Each symbol represents an individual mouse; horizontal bars correspond to mean values of the respective group. Five mice per group were used. This is one representative result of three independent experiments. Statistical significance between groups is indicated above symbols (*P<0.05; **P<0.01).

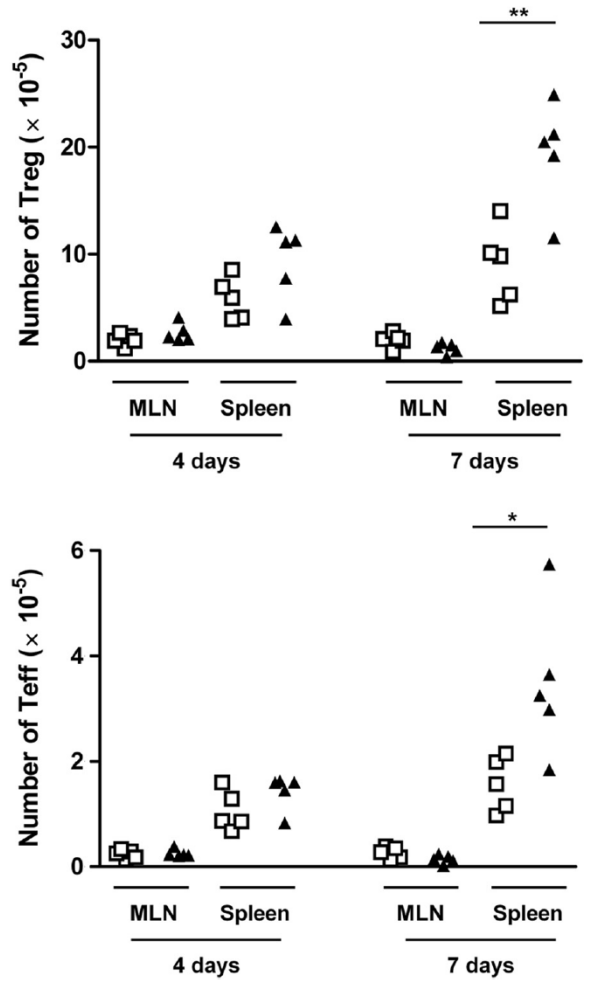

Figure 4 Spleen and MLN Treg and Teff cell numbers. Scatter plots of $CD4^+CD25^+$ Treg ($Foxp3^+$) and Teff ($Foxp3^-$) cell numbers in the MLN and spleen of control (open squares) or *N. caninum*-infected mice (closed triangles), 4 and 7 days upon the i.g. challenge. Each symbol represents an individual mouse; horizontal bars correspond to mean values of the respective group. Five mice per group were used. This is one representative result of three independent experiments. Statistical significance between groups is indicated above symbols (*$P<0.05$; **$P<0.01$).

caninum antigen extracts was confirmed by up-regulated expression of surface MHC class II, CD40 and CD86, detected by using flow cytometry (data not shown). As shown in Figure 5b, antigen-driven T cell proliferation was also more effectively suppressed by Treg from infected mice than by Treg from non-infected controls.

Decreased levels of IFN-γ and also of IL-10 were detected in the supernatants of anti-CD3 mAb-stimulated co-cultures of splenic naïve $CD4^+CD25^-$ T cells when co-cultured with splenic $CD4^+CD25^+$ T cells from infected and control mice. $CD4^+CD25^+$ T cells from both *N. caninum*-infected mice and non-infected controls, efficiently suppressed the production of IFN-γ and IL-10 to levels similar to those of non-stimulated cells. The equivalent suppression of cytokine production by Treg from both groups, when a higher suppressive activity on T cell proliferation was observed for Treg from infected mice might result from the high Treg: T responder ratio (1:1) which may have prevented differences to show up. Interestingly, in similar co-cultures of cells sorted from the MLN, $CD4^+CD25^+$ T cells from the MLN of infected mice suppressed more efficiently the production of IFN-γ by MLN responder cells than $CD4^+CD25^+$ T cell counterparts from non-infected controls. In contrast, in the latter cultures, no suppression of IL-10 production was observed likely because IL-10 levels in the supernatants of stimulated cultures were no significantly different from the ones found in non-stimulated cultures (Figure 6). No differences were found in the levels of IL-4 in any of the conditions tested (data not shown). Altogether, these results show that $CD4^+CD25^+$ T cells from *N. caninum*-infected mice display an enhanced suppressive activity when compared with the equivalent T cell population from non-infected controls.

Discussion

Although *N. caninum* can infect its natural hosts through the GI tract [7], very little is known about the local immune response in the intestinal mucosa and associated lymphoid tissue. We have previously reported that neosporosis could be established in mice i.g. challenged with *N. caninum* tachyzoites [11]. Although this parasitic form may present antigenic differences from oocysts and sporozoites, this model may nevertheless better mimic the natural infection route in horizontally transmitted neosporosis, than the intraperitoneal or subcutaneous routes. The i.g. infection model was used here to study the immune response elicited in the intestinal mucosa and MLN. In infected mice, tachyzoites could be detected within the intestinal tissue early after the parasitic challenge. Also soon after infection, parasitic DNA was detected in the MLN of one infected mouse. This might explain the stimulatory effect on MLN cDC and pDC observed in the infected mice, and their increased expression of IL-12, a key cytokine in mediating host immune protection against *N. caninum* infections [14-16]. On the other hand, as DC have been shown to help systemic dissemination of *N. caninum* [21] and of the closely-related protozoan *T. gondii* [22,23], it would be interesting to determine whether DC may transport the parasites from the gut to the MLN, contributing for parasite dissemination within the host. The detection of *N. caninum* DNA in the MLN of an i.g.-infected mouse as early as 6 h after infection might support this hypothesis, though further studies must be carried out to confirm such a role of DC in this infection model. As

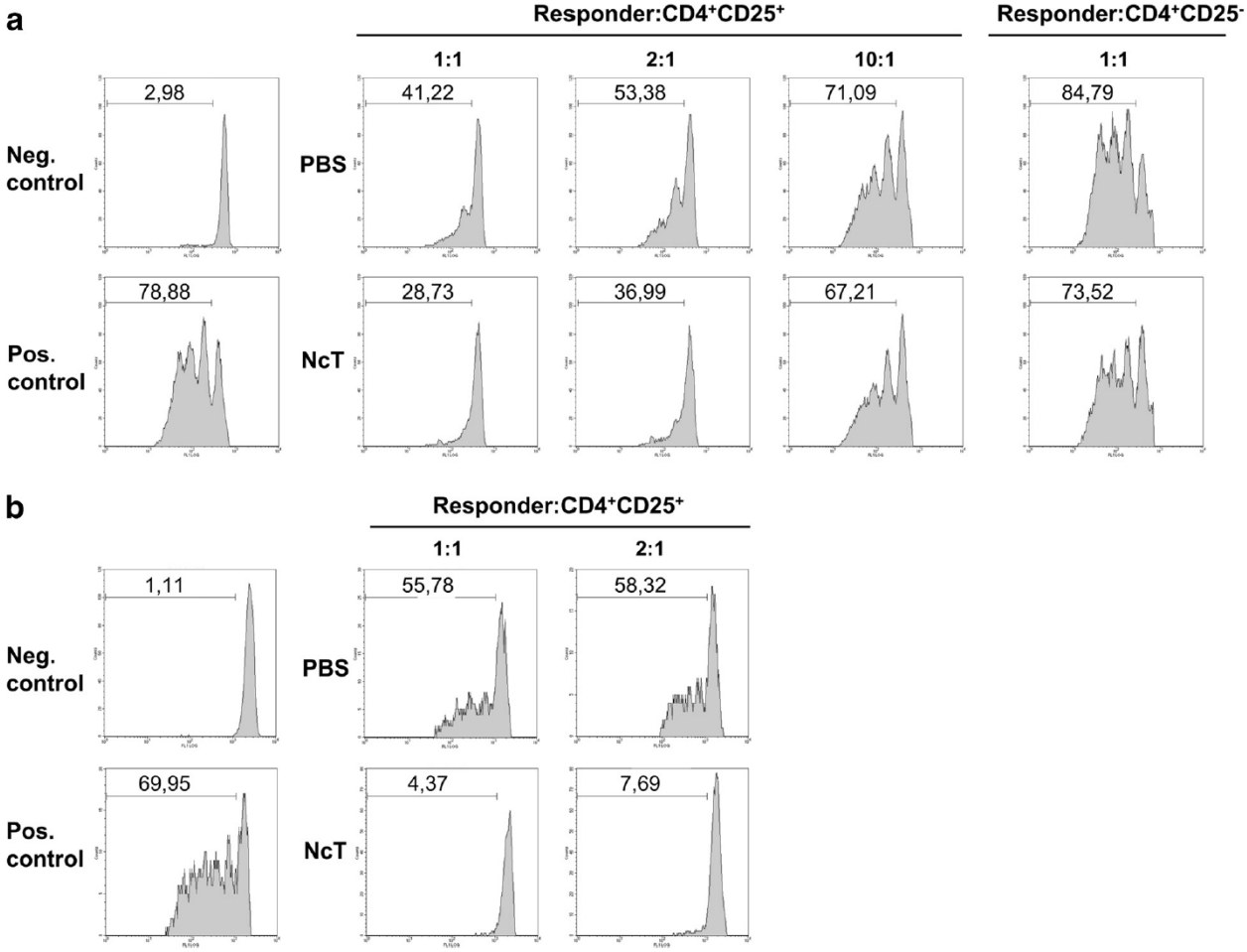

Figure 5 High suppressive activity of Treg from *N. caninum*-infected mice. (a) Flow cytometric evaluation of anti-CD3 mAb (1 μg/mL) induced proliferative response of 2.5×10^4 CFSE-labelled naïve $CD4^+CD25^-$ T (responder) cells cultured for 72 h with 10^5 irradiated APC/well, in the absence (Pos. control) or presence of $CD4^+CD25^+$ T cells obtained from control mice or mice infected with *N. caninum*. Neg. control corresponds to responder cells with no mAb added. Histograms correspond to 1:1, 2:1 and 10:1 responder: $CD4^+CD25^+$ cell ratios or 1:1 responder: $CD4^+CD25^-$ T cell ratio, as indicated. **(b)** Flow cytometric evaluation of *N. caninum* antigen-induced proliferative response of 2.5×10^4 CFSE-labelled splenic $CD4^+CD25^-$ T (responder) cells obtained from 7 day-infected mice, cultured for 72 h with 10^5 antigen-loaded BMDC/well, in the absence (Pos. control) or presence of $CD4^+CD25^+$ T cells obtained from control mice or mice infected with *N. caninum*. Non-specific proliferation control (Neg. control) corresponds to responder cells cultured with LPS (50 ng/mL) activated BMDC, without *N. caninum* antigen. Histograms correspond to 1:1 and 2:1 responder: $CD4^+CD25^+$ cell ratios, as indicated. Numbers within histograms correspond to the percentages of cells that divided at least once. $CD4^+CD25^+$ cells added in each condition were sorted from pooled splenic cells of 5 mice per group. Results are a representative example out of three **(a)** or one out of two **(b)** independent experiments.

parasite DNA was detected in the brain of infected mice 21 days upon infection, this confirmed previous results showing that *N. caninum* tachyzoites might cross the intestinal epithelial barrier and disseminate to other organs [11]. Lack of evident signs of disease in the infected mice indicates that mice are able to control neosporosis established by the i.g. route. Nevertheless, it cannot be excluded that this control might in part be due to a low number of parasites successfully crossing the intestinal epithelial barrier.

Our results, by showing that MLN DC produce IL-12 in response to *N. caninum* infection are in agreement with our previous observation that both cDC and pDC produced IL-12 in the spleen of mice infected i.p. with *N. caninum* tachyzoites [14] and with other reports showing in vivo [15] and in vitro [24,25] production of this cytokine upon DC stimulation with this parasitic form. Both cDC and pDC populations were demonstrated to be early sources of IL-12 in mice infected with *T. gondii* [26,27] and their importance for host protection against this parasite has been recently highlighted [28,29]. A similar protective role of these cell populations may also be important for host resistance against *N. caninum* infection. Our results indicate that such a

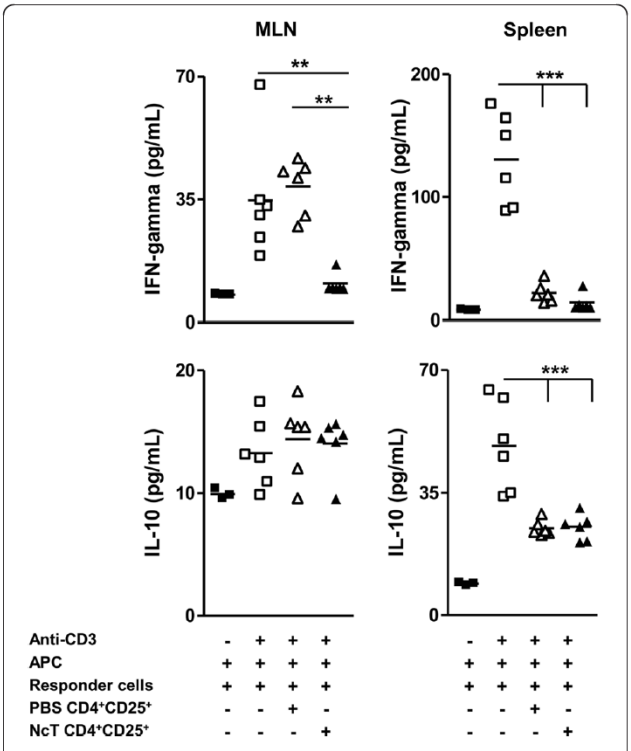

Figure 6 Suppression of cytokine production by splenic and MLN Treg. IFN-γ and IL-10 cytokine concentration in the supernatants of MLN or splenic naïve 2.5×10^4 $CD4^+CD25^-$ T cells cultured for 72 h with anti-CD3 mAb (1 μg/mL) and 10^5 APC, alone or in the presence of 1:1 $CD4^+CD25^+$ MLN or splenic T cells from control or *N. caninum*-infected mice, as indicated. Each symbol represents an individual culture well. Horizontal bars represent the mean values of the respective group. $CD4^+CD25^+$ cells added in each condition were sorted from pooled MLN or splenic cells of 5 mice per group, as indicated. Results are a representative example out of three independent experiments. Statistical significance among groups was determined by one-way ANOVA, followed by a Bonferroni post-test, and is indicated above symbols (**$P<0.01$; ***$P<0.001$).

protective immune response may be triggered already at the mucosal immune system in hosts challenged with this parasite in the GI tract. The IL-12 production detected in the MLN of the infected mice may contribute for the differentiation of IFN-γ-producing $CD8^+\alpha\beta TCR^+$ IEL, found in higher proportions in these mice. Previous works have reported the importance of MLN [30], and of MLN DC in particular [31], in generating $CD8^+\alpha\beta TCR^+$ IEL. Primed IEL have been shown to mediate protective immunity to oral *T. gondii* infection in adoptive cell transfer experiments [32,33]. Therefore it could be expected that these cells would have a similar role in *N. caninum*-infected mice.

In bovine neosporosis, the study of $CD8^+$ T cells mainly addressed their possible participation in the immune response associated with foetal loss [34-36]. Nevertheless, $CD8^+$ T cells have been extensively demonstrated to have a host protective role against parasitic infections [37], including neosporosis [34]. The production of IFN-γ by $CD8^+$ T cells, which was also detected in calves experimentally infected with *N. caninum* [35], is an important mechanism in their host protective role against parasite infections [38-42]. It has been particularly shown that $CD8^+$ T cells were the main early producers of IFN-γ in murine toxoplasmosis and were involved in resistance to acute primary infection [43]. Our results indicate that in murine i.g.-established neosporosis, $CD8^+$ T cells are also early major producers of the protective cytokine IFN-γ. These cells were found elevated in the MLN of infected mice but also in the spleen. It would be interesting to determine whether these cells were locally activated in the spleen and identify the antigen-presenting cells responsible for this activation. Elevated proportions of IFN-γ-producing $CD4^+$ T cells were detected later than $CD8^+$ counterparts in the spleen and MLN of infected mice and not at all in the intestinal epithelium. A predominant response of $CD8^+$IFN-γ^+ IEL as compared to $CD4^+$ counterparts has been also observed in mice orally infected with *T. gondii* [44]. Why $CD8^+$ T cells apparently respond faster than $CD4^+$ T cells in the gut mucosa and draining lymph nodes remains to be determined. A hypothesis worth to explore could be that *N. caninum* differentially affect the class I vs class II major histocompatibility complex antigen presentation pathways.

Production of IL-12 by MLN DC elicited in mice orally infected with *T. gondii* oocysts was shown to depend on bacterial translocation, promoted by the inflammatory reaction in the gut that followed the oocyst administration [45]. As no evidence of significant intestinal inflammation was found in the *N. caninum*-infected mice, IL-12 production may depend mostly on the parasitic antigens. Production of the pro-inflammatory cytokine IL-17 was associated with intestinal inflammatory pathology [46-48]. Our results, by showing that production of this cytokine was not detected in elevated proportions of IEL or MLN T cells, are thus in agreement with the lack of evident inflammation in the gut of the infected animals.

Control of microbial induced inflammation, including that caused by protozoans, largely depends on the action of Treg cells [49,50]. The observation reported here, by showing a high suppressive function of Treg obtained from *N. caninum*-infected mice may provide an additional explanation for the success of *N. caninum* in colonizing its natural hosts, where it can persist in a symptomless condition [51]. This highly suppressive function was more evident when antigenic instead of polyclonal stimulation was used in the Treg in vitro suppression assay of T cell proliferation. It is thus plausible that *N. caninum*, as demonstrated for other protozoan

parasites [52], might manipulate natural Treg function in order to favour its persistence within the host. Interestingly, a recent report on persistent *Salmonella* infection showed that Treg suppressive potency decreased from the acute to the chronic phase, significantly affecting bacterial burden [53]. It would be worthwhile examining if the suppressive function of Treg later in *N. caninum* infection could be diminished when the acute phase of infection is overcome. Here, the immunosuppressive function was revealed by the inhibition of both in vitro T-cell proliferation and cytokine production. Curiously, no significant suppression of IFN-γ production was observed in co-cultures of Treg from the MLN of non-infected mice and MLN responder cells whereas the Treg spleen counterparts were highly suppressive. Particular environmental conditions of the MLN [54] might have conditioned both Treg and T conventional cells responsiveness, as may be suggested by the lower cytokine production of the MLN responder cells upon induction with anti-CD3 and irradiated APC, when compared with similarly stimulated spleen counterparts. As a decrease of spleen and MLN $CD8^+IFN\text{-}\gamma^+$ T cells proportions and numbers to underneath basal levels by day 7 of infection was observed, it would be interesting to determine whether it may reflect Treg function. In fact, other reports on apicomplexan parasite infections show that Treg, apart from suppressing $CD4^+$ T cell proliferation and cytokine production, similarly affect $CD8^+$ T cells [55,56]. It would be also interesting to assess whether such a high suppressive activity could be induced in mice infected by using other parasite administration routes. Moreover, it would be interesting to evaluate whether *N. caninum* infection would affect Treg suppressive activity along the gestational period and its influence in the cytokine environment, since higher levels of IFN-γ were detected in infected pregnant dams carrying live foetuses [57].

The suppressive activity of T regulatory cells may pose an additional difficulty to overcome infection by means of vaccination, as previous remarked [58]. As a preferential involvement of $CD8^+$ T cells in the mucosal immune response to *N. caninum* was shown herein, the stimulation of parasite-specific effector and memory $CD8^+$ T cell responses at mucosal sites may be a privileged target to achieve in vaccination against horizontally transmitted neosporosis.

In conclusion, intragastric infection of C57BL/6 mice with *N. caninum* tachyzoites preferentially activates mucosal and splenic $CD8^+$ T cells, resulting in the production of the host protective cytokine IFN-γ. Nevertheless, the highly suppressive Treg present in the spleen of *N. caninum*-i.g.-infected mice may contribute to the establishment of a chronic infection.

Additional files

Additional file 1: Detection of *N. caninum* in the intestinal tissue of mice infected by the i.g. route. Representative images showing a *N. caninum* tachyzoite in the murine intestinal tissue **(a** and **b)**, 12 h upon i. g. infection, detected by immunohistochemistry. *N. caninum* tachyzoite (brown colour, denoted by arrow). The selected area in **(a)** is presented at higher magnification in **(b)**. Bar=100 μm. Results are representative of data from two independent experiments.

Additional file 2: Proportions of Treg within splenic and MLN $CD4^+$ $CD25^+$ T cells. Flow cytometry analysis of intracellular Foxp3 expression in splenic and MLN $CD4^+$ T cells from C57BL/6 mice, 4 and 7 days after i. g. challenge with PBS or 5×10^7 *N. caninum* tachyzoites (NcT), as indicated. **(a)** Gating of $CD4^+CD25^+$ and of $CD4^+CD25^-$ T cells. **(b)** Numbers within dot plots correspond to mean ± one SD of Treg ($Foxp3^+$ cells) frequency within gated $CD4^+CD25^+$ T cell population. **(c)** Numbers within dot plots correspond to mean ± one SD of the frequency of $CD4^+$ $CD25^-$ T cells expressing Foxp3, in the spleen of non-infected or infected mice, 7 days upon the parasitic challenge. In each panel, results are of a representative experiment out of at least three independent experiments (n=5 in each group). Statistical significance between groups in panel c is indicated (*P<0.05). No statistically significant differences were observed in the frequencies of Treg and Teff between control and infected mice.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AC and MV conducted and supervised the experiments, analysed the data, and wrote the manuscript. AF, LT and AR assisted in the experimental design and data analysis, and contributed to the interpretation of results and manuscript writing. PF, JD, AAC, and RC conducted the experiments, analysed data and contributed to the interpretation of results. JM participated in the experiments on Figures 1 and 2 and contributed to the analysis and interpretation of results therein. AR participated in data acquisition in the experiments involving mice. All authors read and approved the final manuscript.

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Annex 2

Protective effect of intranasal immunization with *Neospora caninum* membrane antigens against murine neosporosis established through the gastrointestinal tract

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IMMUNOLOGY ORIGINAL ARTICLE

Protective effect of intranasal immunization with *Neospora caninum* membrane antigens against murine neosporosis established through the gastrointestinal tract

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Summary

Neospora caninum is an Apicomplexa parasite that in the last two decades was acknowledged as the main pathogenic agent responsible for economic losses in the cattle industry. In the present study, the effectiveness of intranasal immunization with *N. caninum* membrane antigens plus CpG adjuvant was assessed in a murine model of intragastrically established neosporosis. Immunized mice presented a lower parasitic burden in the brain on infection with 5×10^7 tachyzoites, showing that significant protection was achieved by this immunization strategy. Intestinal IgA antibodies raised by immunization markedly agglutinated live *N. caninum* tachyzoites whereas previous opsonization with IgG antibodies purified from immunized mice sera reduced parasite survival within macrophage cells. Although an IgG1 : IgG2a ratio < 1 was detected in the immunized mice before and after infection, indicative of a predominant T helper type 1 immune response, no increased production of interferon-γ was detected in the spleen or mesenteric lymph nodes of the immunized mice. Altogether, these results show that mucosal immunization with *N. caninum* membrane proteins plus CpG adjuvant protect against intragastrically established neosporosis and indicate that parasite-specific mucosal and circulating antibodies have a protective role against this parasitic infection.

Keywords: antibody responses; CpG DNA; mucosal immunity; mucosal vaccines; parasitology.

Introduction

Neospora caninum is an Apicomplexa parasite initially described as the causative agent of neuromuscular disease in dogs.[1] Although canids have been identified as the definitive hosts of *N. caninum*, this parasite can infect a wide range of intermediate hosts including bovines.[2] Infected cattle have increased incidence of abortion, which, together with the high efficiency of vertical transmission, makes neosporosis responsible for severe economic losses.[3] Therefore, effective control methods that could prevent parasite spread are necessary. Lack of intervention carries too great a risk and a test and cull approach, despite its effectiveness, is too expensive. Coccidiostatic treatment also appears to be an expensive option that raises concerns regarding its use in animals

Abbreviations: BMDM, bone marrow-derived macrophages; CpG, oligodeoxynucleotides containing non methylated guanine-*p*-citosine motifs; i.g., intragastric; IgA-CpG, IgA purified from the intestinal lavage fluids of mice from the CpG group; IgA-NcMP/CpG, IgA purified from the intestinal lavage fluids of mice from the NcMP/CpG group; IgG-CpG, IgG purified from the sera of mice from the CpG group; IgG-NcMP/CpG, IgG purified from the sera of mice from the NcMP/CpG group; i.n., intranasal; ILF, intestinal lavage fluids; LCCM, L-929 cell condition medium; mAb, monoclonal antibody; MOI, multiplicity of infection; MLN, mesenteric lymph nodes; NcMP, *Neospora caninum* membrane proteins; NcS, *Neospora caninum* sonicates; PE, phycoerythrin; PerCP-Cy5.5, peridinin-chlorophyll proteins-cychrome 5.5; qPCR, quantitative real-time PCR; RT, room temperature; SD, standard deviation; SEM, standard error of the mean; VLF, vaginal lavage fluids

for human consumption.[4] Vaccination appears to be the best approach to effectively control neosporosis.[5] However, no commercial vaccine is currently available for neosporosis, after the recent withdrawal of Bovilis® Neoguard, which nevertheless had limited efficacy.[3] Therefore, development of a novel vaccine that could prevent this parasitic disease is a pressing necessity.

Attenuated *N. caninum* tachyzoites were successfully used to immunize mice[6–8] or cattle[9] against neosporosis. However, the use of attenuated strains is undesirable because of their short shelf life and the possible regression to a more virulent status.[5] On the other hand, although it was reported that immunization with whole parasite lysates protected mice from *N. caninum* infection or vertical transmission,[10,11] other studies showed that immunization using parasite lysates conferred little protection or even exacerbated the outcome of murine infection[12–14] and failed to prevent vertical transmission in cattle.[15] Recombinant *N. caninum* proteins have also been tested as potential vaccine candidates with promising although variable efficacy.[16–22] Nonetheless, and despite the gastrointestinal mucosa being a natural infection route for *N. caninum*, mucosal (intranasal; i.n.) immunization against neosporosis has been attempted in a limited number of studies that yielded encouraging results.[23–25] Despite the immunization route used therein, the immune response in the mucosae or associated lymphoid tissue was not specifically addressed.

Here, our previously described model of *N. caninum* infection established through the gastrointestinal tract[26,27] was used to assess the protective effect of i.n. immunization against neosporosis by using *N. caninum* membrane proteins (NcMP) as target antigens. Our results show that immunization with NcMP plus CpG adjuvant conferred protection against the parasite infection. Moreover, by showing an *in vitro* effector function of mucosal and circulating antibodies, we provide evidence for a protective role of the humoral immune response against neosporosis.

Materials and methods

Animals

Seven-week-old female C57BL/6 mice were purchased from Charles River (Barcelona, Spain). Animals were kept at the Instituto de Ciências Biomédicas Abel Salazar animal facility throughout the experimental procedures. Interleukin-12 (IL-12)/IL-23 p40$^{-/-}$ C57BL/6 mice 7–11 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the same facility. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92). Authorization for the experiments was issued by the animal welfare section of the competent national board, Direcção Geral de Veterinária (0420/000/000/2008).

Parasites

Neospora caninum tachyzoites (Nc1 isolate) were kept by serial passages in VERO cell cultures, maintained in minimal essential medium containing Earle's salts (Sigma, St Louis, MO), supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria), L-glutamine (2 mM), penicillin (200 IU/ml) and streptomycin (200 g/ml) (all from Sigma), in a humidified atmosphere with 5% CO_2 at 37°. Tachyzoites were maintained until 80% destruction of the host cell monolayer and were isolated as previously described.[26] Briefly, free parasites and adherent cells were recovered using a cell scraper and centrifuged at 1500 ***g*** for 15 min. The pellet was passed through a 25-G needle and then washed three times in PBS by centrifugation at 1500 ***g*** for 15 min. The resulting pellet was resuspended and passed through a PD-10 desalting column, containing Sephadex™ G-25M (GE Healthcare, Freiburg, Germany). Tachyzoite concentration was determined in a haemocytometer. In these experiments the parasites used underwent fewer than 15 *in vitro* passages from the original ATCC vial. The viability of the used inocula was confirmed in IL-12/IL-23 p40$^{-/-}$ mice that were earlier shown to be highly susceptible to neosporosis.[28]

Preparation of whole tachyzoite lysates and cell-membrane extracts

The NcMP were extracted by using a modification of a previously described method.[29,30] Briefly, free tachyzoites were resuspended in PBS containing 0·75% Triton X-114 (Sigma), incubated for 10 min on ice and centrifuged at 10 000 ***g*** for 30 min at 4°. The supernatant was recovered and placed in a water bath at 30° for 3 min. The procedure was repeated and the supernatant was centrifuged at 1000 ***g*** for 3 min at room temperature. The aqueous phase was discarded and the NcMP were precipitated with the addition of absolute ethanol, vortexed vigorously for 15 seconds and incubated for 1 hr on ice. The samples were centrifuged at 12 000 ***g*** for 20 min at 4° and the resulting pellet was dried, resuspended in PBS and stored at −20°. Whole *N. caninum* lysates were prepared by disruption of tachyzoites following sonication (26 cycles of 15 seconds at 100 W) with a Branson cell disrupter model W 185 D in an ice bath. The obtained *N. caninum* sonicates (NcS) were sequentially passaged through 0·45-μm and 0·2-μm pore-size filters and stored at −20°. Quantification of NcMP or NcS was performed using the Lowry protein assay. SDS–PAGE was performed following each protein extraction to determine and

confirm the protein migration profile. Briefly a discontinuous SDS–PAGE (4–10% acrylamide) was loaded with 10 μg NcMP or NcS, previously heated at 95° for 5 min, and electrophoresis was carried out at a 25-mA constant current. Protein migration profiles were visualized using silver nitrate staining.

Electrophoretic analysis of NcMP

Neospora caninum tachyzoite membrane proteins were prepared for their use as target antigens in mucosal immunization. After extraction, prepared proteins were analysed by SDS–PAGE under reducing conditions and the protein migration profile was determined. As shown in the Supplementary material, Fig. S1, the membrane protein extraction displayed an enrichment of the proteins with molecular weights of approximately 55 000, 35 000 and 29 000, as compared with the proteins obtained in NcS preparations. Additionally, the extraction protocol yielded proteins with estimated molecular weights of 39 000 and 17 000 that were not visible in the NcS gel lane.

Immunizations and tissue sample collection

Eight-week-old female mice were used in three independent experiments with random distribution into four groups per experiment. The immunizations and procedures for collection of serum or vaginal or intestinal lavage fluids (VLF and ILF, respectively) are schematically described in the Supplementary material, Fig. S2. Mice were immunized i.n. at day zero under light isoflurane anaesthesia with 20 μl of PBS containing 30 μg NcMP (NcMP group) or 30 μg NcMP plus 10 μg CpG 1826 VacciGrade (Invivogen, San Diego, CA) (NcMP/CpG group). Sham-immunized control mice were treated with PBS alone (PBS group) or with PBS containing 10 μg CpG 1826 VacciGrade (CpG group). The immunization procedure was repeated 3 weeks after the first immunization. At 6 weeks, all mice were challenged intragastrically (i.g.) with 5×10^7 freshly isolated *N. caninum* tachyzoites as previously described.[26] At 7 weeks, mice were killed by cervical dislocation and spleens and mesenteric lymph nodes (MLN) were aseptically removed for analysis of the immune response, while the brains were collected and stored at −20° for DNA extraction. One week after the boost immunization and 1 week after infection, serum was collected from all mice from the the submandibular vein for detection of *N. caninum*-specific IgG. At 4 and 7 weeks after the first immunization, vaginal and intestinal lavages were performed, respectively, for detection of *N. caninum*-specific IgA. The total number of mice used in the three experiments was 14 in the PBS and NcMP/CpG groups and 13 in the CpG and NcMP groups. Mice similarly immunized with NcMP/CpG or treated with CpG alone ($n = 6$ for both groups) were kept for 4 months after the boost immunization and then killed for analysis of intestinal IgA.

Antibody detection

Serum IgG1 and IgG2a antibodies specific for NcMP were quantified by ELISA. Briefly 96-well plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4° with NcMP diluted in PBS at a concentration of 5 μg/ml. All the wells were saturated with 2% BSA (Sigma) in TST buffer (150 mM NaCl, 10 mM EDTA and 0·05% Tween 20, pH 8) for 1 hr at room temperature. Serum samples were serially diluted in 1% BSA TST buffer and incubated for 1 hr at room temperature, followed by washing and addition of alkaline phosphatase-coupled goat anti-mouse IgG1 or IgG2a monoclonal antibodies (mAb) (Southern Biotechnology Associates, Birmingham, AL) and incubation for 1 hr at room temperature. After washing, the specifically bound antibodies were detected by adding the *p*-nitrophenyl phosphate (Sigma) substrate solution and on development the reaction was stopped by the addition of 0·1 M EDTA, pH 8 solution. The absorbance was measured at 405 nm, subtracting for each well the value of the absorbance at 570 nm. The antibody titres were expressed as the $\log_{10}$ value of the reciprocal highest dilution with an absorbance higher than the value of the control (no serum added). IgA antibodies specific for NcMP were quantified by ELISA as described above, using alkaline phosphatase-coupled goat anti-mouse anti-IgA mAb (Southern Biotech).

Purification of serum IgG antibodies and mucosal IgA

Mouse serum samples and ILF collected on the day of euthanasia were, respectively, used for IgG and IgA purification. Pooled sera collected from mice of the NcMP/CpG and CpG groups were used to purify IgG antibodies by using a HiTrap Protein G HP purification column (GE Healthcare), according to the manufacturer's instructions. Recovered antibodies were buffer-exchanged against sterile PBS to a final concentration of 4·5 mg/ml as determined by Lowry protein assay and stored at −20°. The purified IgG fractions obtained from the sera of CpG or NcMP/CpG groups were, respectively, designated as IgG-CpG or IgG-NcMP/CpG. The NcMP-specific antibody titres of the IgG-CpG and IgG-NcMP/CpG preparations were below the detection limit and $1{\cdot}559 \times 10^9$, respectively, as determined by ELISA.

To obtain IgA antibodies, pooled ILF were passed through a 20-μm pore-size filter before being introduced in a Protein L/Agarose (Invivogen) column. Antibody purification was carried out according to the manufacturer's instructions. Recovered antibodies were buffer-exchanged against sterile PBS, and stored at −20°. The purified IgA

fractions obtained from the ILF of the CpG or NcMP/CpG groups were, respectively, designated as IgA-CpG or IgA-NcMP/CpG. The total IgA titres for the IgA-CpG and IgA-NcMP/CpG preparations were 657 648 and 786 788, respectively, and were normalized to 650×10^3 for further use. The NcMP-specific IgA titres of the IgA-CpG and IgA-NcMP/CpG preparations were below the detection limit and 8995, respectively.

Antibody-binding and parasite-agglutination assays

To evaluate the ability of antibodies present in IgG-CpG, IgA-CpG, IgG-NcMP/CpG or IgA-NcMP/CpG to bind *N. caninum*, different dilutions of these preparations were incubated with 1×10^6 tachyzoites, for 25 min on ice. Detection of bound antibodies was made by using flow cytometry, for which parasites were further incubated with polyclonal anti-IgG antiserum, FITC-conjugated (Southern Biotech), or with anti-IgA FITC-conjugated (BD Biosciences Pharmingen, San Diego, CA) mAb (clone C10-3) for 25 min on ice and then washed with PBS containing 1% BSA and 10 mM sodium azide. Parasite samples were analysed in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter, Miami, FL). The collected data files (100 000 events per sample) were converted for analysis with the CellQuest software, v3.2.1f1 by using FACS convert, v1.0 (both from Becton Dickinson, San Jose, CA). Agglutination assays were performed by incubating 1×10^6 tachyzoites with either IgA-CpG or IgA-NcMP/CpG or PBS alone for 1 hr at 4°. After incubation, smears of each sample were prepared on microscope slides that were fixed in cold methanol for 5 min. Samples were then stained with Hemacolor 2 and 3 (Merk, Darmstadt, Germany) according to the manufacturer's instructions. Mounted slides were observed in a light microscope and 20 micrographs at 200 × and 400 × magnification were taken (Leica Qwin plus v3.5.1 Software, Leica Microsystems, Wetzlar, Germany) as a representative display of each slide. The number and size of parasite clusters were analysed using ImageJ software (Version 1.47, National Institutes of Health, Bethesda, MD).

Intracytoplasmic staining

For intracellular cytokine detection by flow cytometry, spleens and MLN were aseptically removed from the killed infected mice, homogenized in Hanks' balanced salt solution (Sigma) and red blood cells were lysed. The remaining cells were counted and plated in round-bottom 96-well plates (Nunc), at a concentration of 1×10^6 cells/ml in RPMI-1640 (Sigma) supplemented with 10% fetal calf serum (PAA Laboratories), HEPES (10 mM), penicillin (200 IU/ml) and streptomycin (200 g/ml) (all from Sigma), β-mercaptoethanol (0·1 mM) (Merk) (RPMI-1640 complete medium). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37° for 5 hr under stimulation with 20 ng/ml PMA (Sigma), 200 ng/ml ionomycin (Merk) and 10 ng/ml brefeldin A (Epicentre Biotechologies, Madison, WI). Then, cells were recovered and non-specific antibody binding was prevented by the pre-incubation with anti-FcγR mAb followed by incubation with either anti-CD4 peridinin-chlorophyll protein-cychrome 5.5 (PerCP-Cy5.5) -conjugate (clone RM4-5) or anti-CD8 PerCP-Cy5.5-conjugate (clone 53-6.7) mAb (both from BD Biosciences). Following extracellular staining the cells were washed, fixed in 2% formaldehyde, washed again and permeabilized with 0·05% saponin (Sigma)/PBS solution. Intracytoplasmic staining was carried out with anti-interferon-γ (IFN-γ) FITC-conjugate (clone XMG1.2), anti-IL-4 phycoerythrin-conjugate (clone BVD4-1D11) and anti-IL-10 phycoerythrin-conjugate (clone JES5-16E3) (all from BD Biosciences) after pre-incubation of the cells with anti-FcγR mAb. Antibody-labelled cells were analysed in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter). At least 150 000 events were acquired per sample. The collected data files were converted using FACS convert, v1.0 (Becton Dickinson) and analysed using Cell Quest software, v3.2.1f1 (Becton Dickinson).

In vitro *cell cultures and cytokine detection*

To assess *in vitro* cytokine production by NcMP-stimulated spleen and MLN cells, 5·0-ml aliquots of cell suspensions prepared as described above for intracytoplasmic staining, by homogenizing these organs in Hanks' balanced salt solution (Sigma) followed by red blood cell lysis, were layered onto 2·5 ml of a polysucrose-sodium ditrizoate solution (Histopaque 1083®, Sigma) and centrifuged at 800 ***g*** for 20 min at room temperature. Mononuclear cells collected from the medium–Histopaque interface were washed, suspended in RPMI-1640 complete medium, plated ($5{\cdot}0 \times 10^5$/well) in round-bottom 96-well plates, and stimulated with NcMP (100 μg/ml) for 5 days at 37° and 5% CO_2. Four animals from each group were used and triplicate wells were set for cells cultured from each animal. The concentrations of IFN-γ and IL-4 in cell culture supernatants were quantified with the Mouse IFN-γ DuoSet® ELISA development system (R&D Systems, Minneapolis, MN) and the IL-4 ELISA Ready-Set-Go!® (eBioscience, San Diego, CA) kits, respectively, both according to the manufacturer's instructions.

Macrophage cell cultures and parasite opsonization survival

Murine bone marrow-derived macrophages were differentiated from bone marrow precursors. The bone marrow-derived macrophage cultures were generated in six-well plates (Nunc) by culturing 5×10^6 cells in 5 ml

RPMI-1640 complete medium supplemented with 10% L-929 cell line conditioned medium and incubated at 37° in a 5% CO_2 humidified chamber. On day 4, the cell culture medium was renewed and 5 ml of fresh medium supplemented with L-929 cell line conditioned medium was added. Differentiated macrophages were harvested on day 7 by gently scraping the wells. The cells were counted and plated in 24-well plates at a concentration of 1×10^6 cells/ml.

Macrophages were then infected at a multiplicity of infection of 1 : 1 with *N. caninum* tachyzoites incubated with IgG-NcMP/CpG or IgG-CpG, as described above, at different dilutions or with untreated parasites as control. Cells were incubated for 6 hr at 37° in a 5% CO_2 humidified chamber.

DNA extraction

DNA from the brain of infected mice or from macrophages infected with the parasite was extracted as previously described.[31] Briefly, brains were weighed and homogenized. Both sample types were incubated overnight at 55° in a solution containing 1% SDS and 1 mg/ml Proteinase K (Sigma). DNA was extracted by the phenol–chloroform (from Sigma and Merk, respectively) method followed by ammonium acetate/ethanol precipitation.

Real-time PCR analysis

The parasite burden in the brain of infected mice and macrophage cell cultures was assessed by a quantitative real-time PCR (qPCR) analysis of the parasite DNA performed in a Corbett rotor gene 6000 system (Corbett Life Science, Sydney, NSW, Australia). Brain analysis was performed using a Rotor-Gene probe PCR kit (Qiagen, Hilden, Germany), for the amplification of a 103-bp sequence of the Nc5 region of the *N. caninum* genome using the primers NcA 5′-GCTACCAACTCCCTCGGTT-3′ and NcS 5′-GTTGCTCTGCTGACGTGTCG-3′, both at a final concentration of 0·2 μM, and the fluorescent probe FAM-CCCGTTCACACACTATAGTCACAAACAAAA-BBQ at a final concentration of 0·1 μM (all designed and obtained from TIB-Molbiol, Berlin, Germany). The DNA samples were amplified using the following programme: 95° for 3 min, 95° for 5 seconds, 60° for 20 seconds with fluorescence acquisition. The second and third steps were repeated 50 times. Length of the amplified DNA was confirmed in a 3% agarose gel stained with ethidium bromide. Macrophage samples were analysed using Express Sybr green ER qPCR supermix universal (Invitrogen), for the amplification of a 337-bp sequence of the Nc5 region of the *N. caninum* genome using the primers Np21plus 5′-CCCAGTGCGTCCAATCCTGTAAC-3′ and Np6plus 5′-CTCGCCAGTCAACCTACGTCTTCT-3′ (both from TIB-Molbiol), both at a final concentration of 0·25 μM. The DNA samples were amplified using the following programme: 95° for 10 min, 95° for 30 seconds, 63° for 20 seconds, 72° for 45 seconds with fluorescence acquisition, the second, third and fourth steps were repeated 45 times. A melting curve was performed in each run to access the PCR-amplified fragments: from 65° to 95°, with increments of 1° for 5 seconds. In all runs, the parasite burden was determined by interpolation of a standard curve, ranging from 10^1 to 10^{-4} ng of DNA extracted from *N. caninum* tachyzoites included in each run and the data were analysed using the Rotor gene 6000 software v1.7 (Corbett Life Science).

Statistical analysis

Statistical analyses were performed using GraphPad software (Version 5.0, GraphPad Software, Inc. La Jolla, CA). In the scatter dot graphs the mean for each group was displayed as a horizontal line. Column graphs are represented showing the mean plus one standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance with Newman–Keuls *post-hoc* analysis.

Results

Intestinal mucosa IgA produced on immunization binds to and agglutinates *N. caninum* tachyzoites

Production of IgA is the hallmark of specific immune responses at the mucosa.[32] As IgA levels in the small intestine are difficult to ascertain without invasive procedures, NcMP-specific IgA titres were measured in VLF to monitor the effectiveness of the mucosal immunization. As shown in Fig. 1(a), higher titres of antigen-specific IgA were detected in the VLF of mice from the NcMP/CpG group than in the other groups at 7 days after the boosting i.n. immunization. Accordingly, elevated levels of NcMP-specific IgA were detected in ILF of the NcMP/CpG immunized mice, as compared with the other groups studied, 7 days after infection (Fig. 1b). Mice of the NcMP group also presented higher antibody levels in the VLF and ILF than control groups at both assessed time-points, although lower than those of the NcMP/CpG group, so highlighting the adjuvant effect of CpG. NcMP-specific IgA levels in the ILF of non-infected NcMP/CpG-immunized mice were sustained and were elevated 4 months after the boost immunization compared with those of CpG-treated controls (2·62 ± 0·31 mean $\log_{10}$ IgA titre ± 1 SD versus below detection limit values, respectively; $n = 6$/group). The ability of the raised IgA antibodies to bind *N. caninum* tachyzoites was confirmed using flow cytometry. As shown in Fig. 2(a), a significant increase in the mean fluorescence intensity value due to parasite-bound IgA was observed in tachyzoites incubated

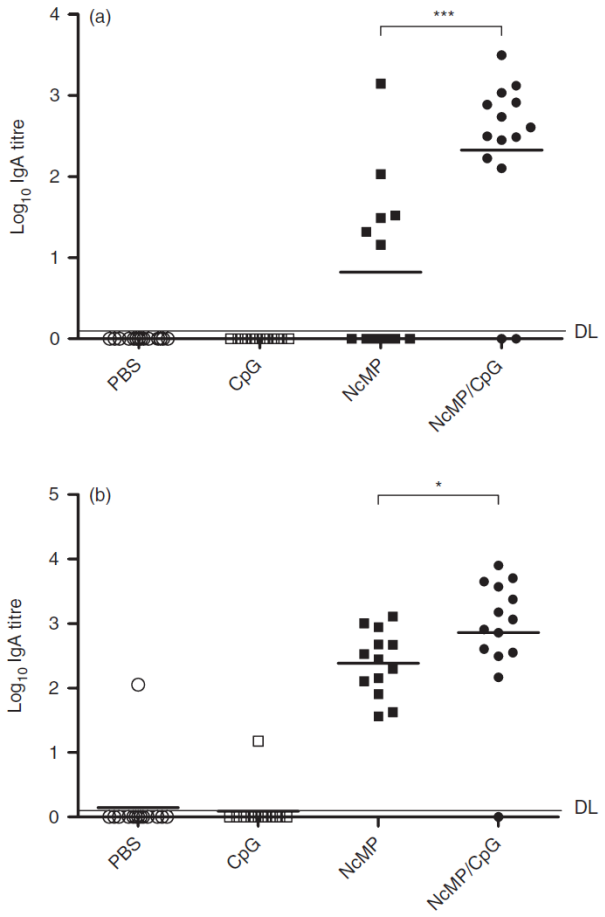

Figure 1. *Neospora caninum* membrane proteins (NcMP)-specific IgA titres detected by ELISA in vaginal and intestinal lavage fluids (VLF and ILF, respectively). IgA titres were determined by ELISA in (a) VLF collected 7 days after the second immunization from mice immunized twice intranasally with NcMP with or without CpG adjuvant (NcMP and NcMP/CpG, respectively) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG); (b) ILF collected from the same groups 7 days after intragastric challenge with 5×10^7 *N. caninum* tachyzoites performed 3 weeks after the last immunizing administration. Results correspond to pooled data of three independent experiments (PBS $n = 14$; CpG $n = 13$; NcMP $n = 13$; NcMP/CpG $n = 14$). Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group [$*P < 0{\cdot}05$; $***$ $P < 0{\cdot}001$; detection limit (DL) is indicated by a horizontal line].

with IgA-NcMP/CpG compared with that of parasites incubated with IgA-CpG, so confirming the specificity of the antibodies raised by immunization. The agglutination of pathogens is one recognized effector function of IgA in the intestinal mucosa, so preventing their attachment to host cells.[33] To assess the agglutinating capacity of the intestinal IgA produced on i.n. immunization, *N. caninum* tachyzoites were incubated with IgA-NcMP/CpG and IgA-CpG and the formation of parasitic agglutinates was assessed by optical microscopy. On incubation, a higher number of parasite clusters and a higher area per cluster (Fig. 2b,c, respectively) were found when the tachyzoites were incubated with IgA-NcMP/CpG than with IgA-CpG or PBS alone. Parasites incubated with IgA-CpG or PBS were mainly observed as single cells, whereas parasites treated with IgA-NcMP/CpG were mainly observed agglutinated in large bodies (see Supplementary material, Fig. S3). Together, these results show that mucosal parasite-agglutinating IgA antibodies are produced on i.n. immunization targeting NcMP.

Serum IgG antibodies elicited on immunization with NcNP plus CpG bind to *N. caninum* and reduce parasite survival in infected macrophages

To determine whether parasite-specific IgG antibodies were also induced by the i.n. immunization, serum samples were analysed for the presence of NcMP-specific antibodies of that isotype. As shown in Fig. 3, the majority of the immunized mice presented high levels of antigen-specific IgG antibodies, detected before and after the i.g. parasitic challenge. All but one mouse treated i.n. with CpG or PBS alone presented no detectable serum IgG antibodies with this specificity by day 7 on the parasitic challenge. Analysis of the IgG isotype profile revealed a mixed IgG1 and IgG2a response in the NcMP and NcMP/CpG groups. However, disparate IgG1/IgG2a ratios were detected in these groups. Whereas in the mice immunized with NcMP alone, this ratio was > 1 before and after infection, a ratio < 1 was observed for the NcMP/CpG group (Fig. 3). As the IgG2a and IgG1 isotypes were, respectively, associated with a T helper type 1 (Th1) and a Th2-type immune response,[34] these results indicate that a predominant Th1-type immune response was induced in the NcMP/CpG group whereas a Th2-type immune response was elicited by NcMP immunization in the absence of adjuvant. To determine the ability of serum IgG produced in the immunized mice to bind *N. caninum* parasites, tachyzoites were incubated with either IgG-NcMP/CpG or IgG-CpG and analysed by flow cytometry. As shown in Fig. 4(a), IgG-NcMP/CpG antibodies bound *N. caninum* tachyzoites more markedly than those in the IgG-CpG preparation.

Neospora caninum is an obligate intracellular parasite and consequently the capacity to infect new cells once inside the host is essential for its survival. Therefore, blocking the infection of new cells could be an important factor for parasite control. To test the effects of the IgG preparations in the capacity of *N. caninum* to survive in the macrophage cell cultures, tachyzoites were incubated with the IgG antibody preparations. The opsonized tachyzoites as well as non-opsonized counterparts were used to challenge macrophage cell cultures for 6 hr, after which the number of parasites therein was evaluated by qPCR. As shown in Fig. 4(b), parasite opsonization with IgG raised by immunization resulted in a dose-dependent

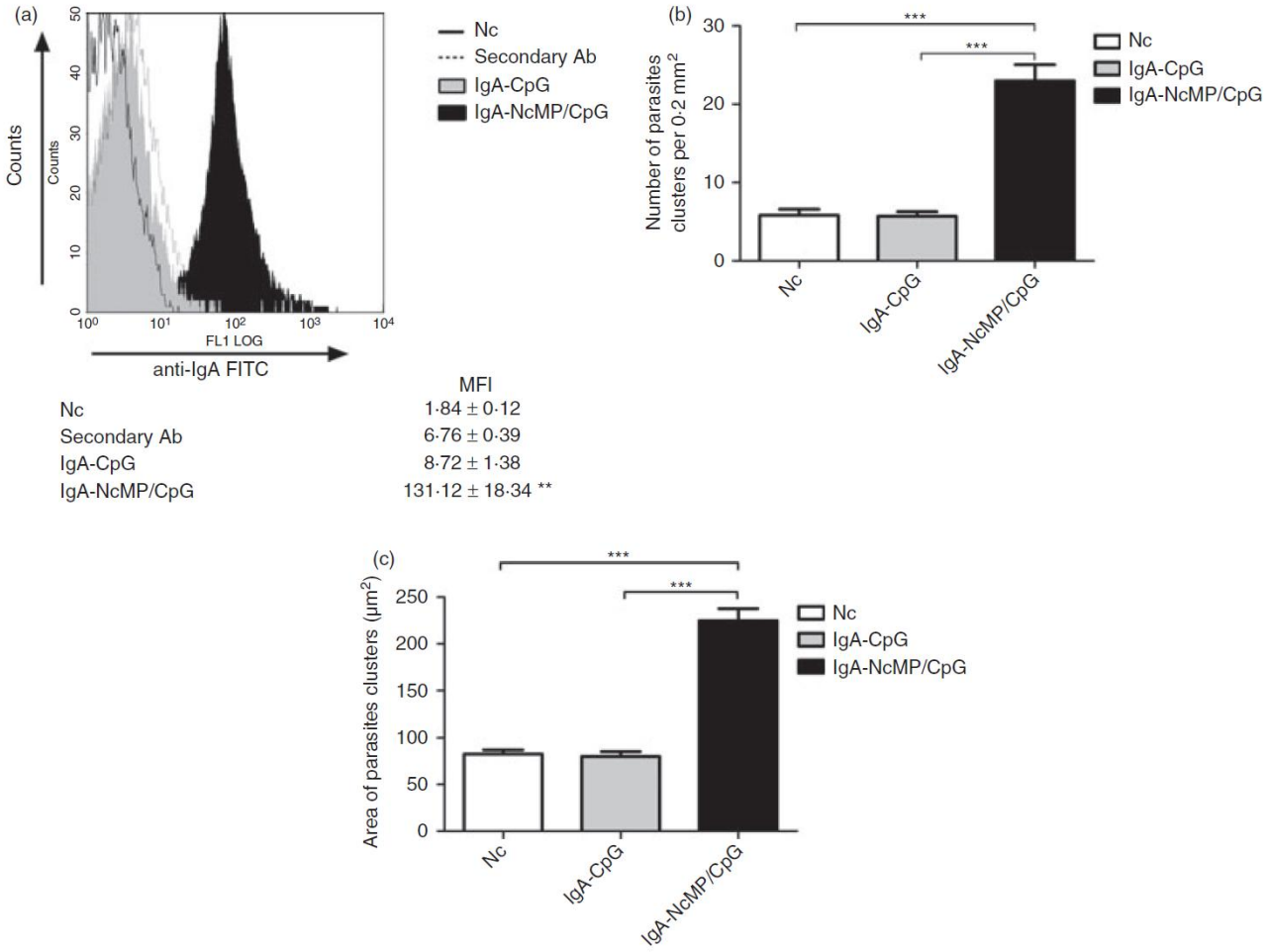

Figure 2. (a) Flow cytometric analysis of anti-IgA-FITC monoclonal antibody (mAb) staining of *Neospora caninum* tachyzoites incubated with intestinal IgA antibodies collected from mice 7 days after intragastric infection with 5×10^7 *N. caninum* tachyzoites 3 weeks after the second of two intranasal immunizations with *N. caninum* membrane proteins (NcMP) plus CpG adjuvant (IgA-NcMP/CpG) or similarly treated with CpG alone (IgA-CpG), or with anti-IgA-FITC alone (Secondary Ab), or untreated (Nc). Histograms are a representative example from three independent experiments with $n = 3$ for each condition. The mean fluorescence intensity (MFI) ± one SD is indicated. Statistical significance of the IgA-NcMP/CpG condition as compared with any of the other conditions is indicated (**$P < 0{\cdot}01$). Parasite agglutination was assessed by Hemacolor staining of tachyzoites incubated with PBS (Nc) or IgA-CpG or IgA-NcMP/CpG fractions for 1 hr. Analysis of the number (b) and size (c) of parasite clusters was made with pooled results of 20 micrographs taken from each condition at a 200 × magnification. Parasite clustering was considered for four or more parasites appearing bound together. Bars correspond to tachyzoites incubated in: PBS alone (Nc), IgA-CpG alone (IgA-CpG) or with IgA-NcMP/CpG (IgA-NcMP/CpG), as indicated. Results are of one representative example out of three independent experiments. Each bar represents the mean value for each group. Error bar = SEM (***$P < 0{\cdot}001$).

reduction in the total number of parasites detected in the cultures.

Protective effect of mucosal immunization against i.g. established neosporosis

Neosporosis is thought to be horizontally transmitted by oocyst ingestion in naturally infected hosts.[35] Therefore, it is conceivable that boosting the immune response in the gastrointestinal mucosa by parasite-specific immunization may increase host resistance against this parasitic disease. To assess whether the immunization procedures used here could be protective, we evaluated by using qPCR the parasitic burden in the brain of mice of the different groups, 7 days after i.g. administration of 5×10^7 *N. caninum* tachyzoites performed 3 weeks after the last immunization. As shown in Fig. 5, a significant reduction of the mean parasitic DNA level in mice of the NcMP/CpG group was detected, as compared with that in control mice, which received CpG or PBS alone. Moreover, the NcMP/CpG group showed the highest number of mice with absent or below detection-limit parasitic DNA ($n = 4$; $n = 5$ and $n = 10$ in the PBS, CpG and NcMP, and NcMP/CpG groups, respectively). Interestingly, the

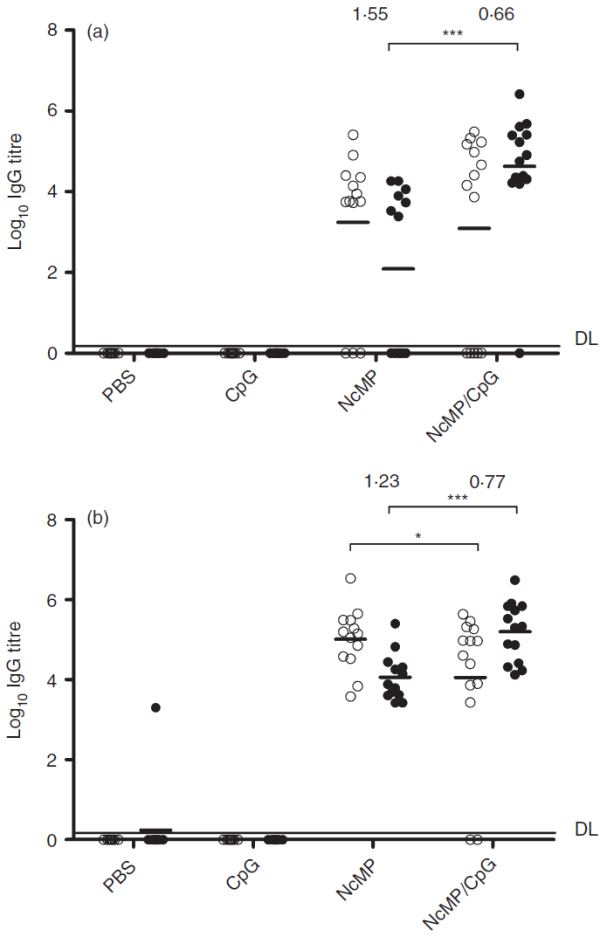

Figure 3. Titres of *Neospora caninum* membrane protein (NcMP) -specific serum IgG1 (open circles) and IgG2a (closed circles) antibodies determined by ELISA (a) 7 days after the second immunization in mice immunized twice intranasally with NcMP with or without CpG adjuvant (NcMP and NcMP/CpG, respectively) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG) or (b) in the same groups, 7 days after an intragastric challenge with 5×10^7 *N. caninum* tachyzoites performed 3 weeks after the last immunizing administration. Numbers above each group represent the IgG1 : IgG2a ratio, calculated with the mean $\log_{10}$ titres for the correspondent IgG isotype. Results correspond to pooled data of three independent experiments (PBS $n = 14$; CpG $n = 13$; NcMP $n = 13$; NcMP/CpG $n = 14$). Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group (*$P < 0{\cdot}05$; ***$P < 0{\cdot}001$; detection limit (DL) is indicated by a horizontal line.

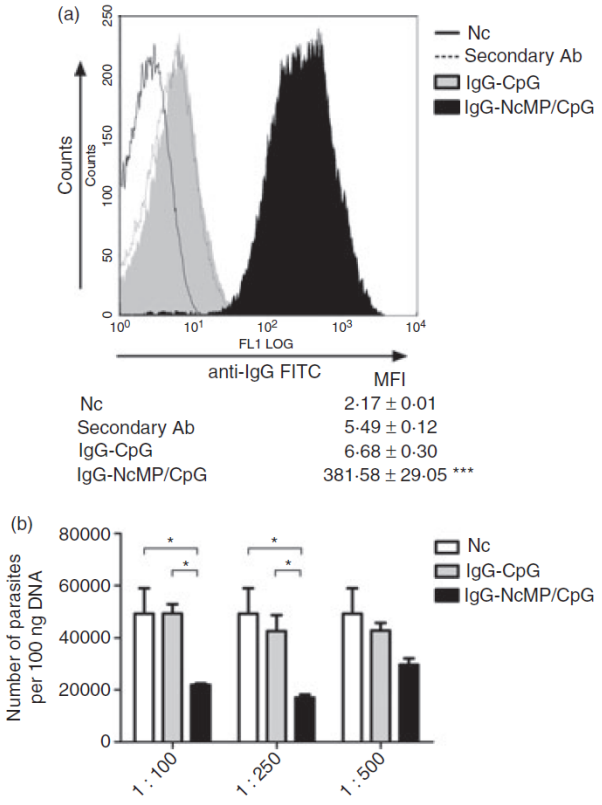

Figure 4. (a) Flow cytometric analysis of anti-IgG-FITC polyclonal antibody staining of *Neospora caninum* tachyzoites incubated with serum IgG antibodies collected from mice 7 days after intragastric infection with 5×10^7 *N. caninum* tachyzoites 3 weeks after being immunized twice intranasally with *N. caninum* membrane proteins (NcMP) plus CpG adjuvant (IgG-NcMP/CpG) or similarly treated with CpG alone (IgG-CpG), or with anti-IgG-FITC alone (Secondary Ab), or untreated (Nc). Histograms are a representative example from one of three independent experiments ($n = 3$ for each condition). The mean fluorescence intensity (MFI) ± one SD is indicated. Statistical significance of the IgG-NcMP/CpG condition as compared with any of the other conditions is indicated (***$P < 0{\cdot}001$). (b) Number of parasites, assessed by quantitative PCR in bone marrow-derived macrophage cell cultures challenged at a multiplicity of infection (MOI) of 1 : 1 for 6 hr with 1×10^6 tachyzoites previously incubated with IgG-CpG or IgG-NcMP/CpG at the indicated dilutions, or untreated parasites (Nc). Results are of a representative example out of three independent experiments. Each bar represents the mean of three wells. Error bar = SEM (*$P < 0{\cdot}05$).

mouse within the NcMP/CpG group showing higher parasitic colonization was the only one presenting no detectable levels of IgA in either VLF or ILF, as shown in Fig. 1. No significant differences were found among any other groups, although the mean parasitic burden detected in the NcMP/CpG group was greatly reduced compared with that of the NcMP group. These results together show that i.n. immunization with NcMP plus CpG adjuvant confers protection against neosporosis established by the gastrointestinal tract.

Cytokine production in the immunized mice

Host production of IFN-γ is associated with resistance to neosporosis whereas production of IL-4 and IL-10 are associated with susceptibility to this infection.[36] Therefore, the frequency and numbers of cells producing these cytokines were assessed in the spleen and MLN of immunized mice and controls, 7 days after i.g. infection. Unexpectedly, no differences were observed among the different analysed groups in the frequencies of splenic $CD4^+$ T cells producing any of these cytokines (Fig. 6a and see Supple-

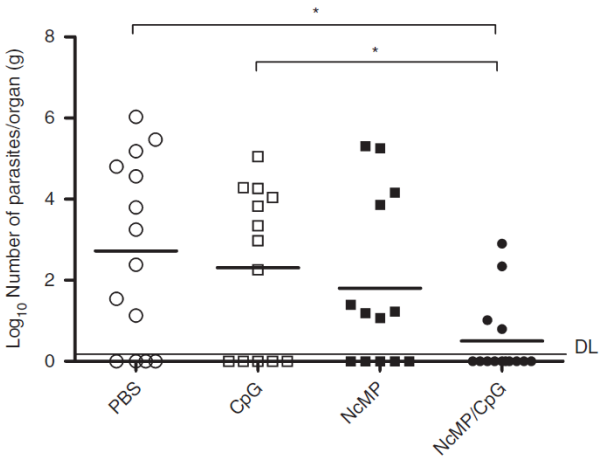

Figure 5. Parasitic load assessed by quantitative PCR 7 days after intragastric challenge with 5×10^7 *Neospora caninum* tachyzoites in the brain of mice previously immunized twice intranasally with *N. caninum* membrane proteins (NcMP) with or without CpG adjuvant (NcMP and NcMP/CpG, respectively) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG). Results are from pooled data of three independent experiments. (PBS $n = 14$; CpG $n = 13$; NcMP $n = 13$; NcMP/CpG $n = 14$). Each dot represents an individual mouse. Horizontal lines correspond to the mean value in each group ($^*P < 0{\cdot}05$); detection limit (DL) is indicated by a horizontal line.

mentary material, Fig. S4). Similarly, no significant differences in the frequency of splenic IFN-γ-producing $CD8^+$ T cells were observed among groups (Fig. 6a). To determine whether, despite being present in similar frequencies to those of controls, $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ T cells of immunized mice could be producing higher amounts of this cytokine, the mean fluorescence intensity due to the IFN-γ staining was assessed in these lymphocyte populations. Results shown in Fig. 6(a) indicate that this is not the case, as mean fluorescence intensity values detected among all assessed groups did not significantly vary. Nevertheless, and surprisingly, lower numbers of splenic $CD4^+$ IFN-γ^+ cells were observed in the NcMP/CpG group compared with the respective CpG control group. Similarly, lower numbers of $CD8^+$ IFN-γ^+ cells were observed in the NcMP/CpG group, although not reaching statistical significance (Fig. 6b). The NcMP/CpG group also presented significantly lower numbers of total $CD4^+$ and $CD8^+$ T cells, compared with the CpG and NcMP groups (Fig. 6c). Similar analyses to all those performed in the spleen were carried out in the MLN that did not show any significant differences among the analysed groups (data not shown). Moreover, no significant differences among groups were observed in the levels of IFN-γ or of IL-4 detected in cell culture supernatants of NcMP-stimulated splenocytes collected from the different analysed mice, 7 days after infection (see Supplementary material, Fig. S4). Cytokine levels in similarly stimulated MLN cell cultures were consistently found near or below detection limits (data not shown). Taken together, these results indicate that in the NcMP/CpG group, the cellular immune response elicited on parasitic challenge was comparable to that of non-immunized PBS-treated infected mice and less noticeable than in the mice that received CpG adjuvant or NcMP alone.

Discussion

We show here that mice i.n. immunized with membrane proteins extracted from *N. caninum* tachyzoites plus CpG adjuvant presented a lower parasitic burden in the brain when infected i.g. with *N. caninum* tachyzoites than controls receiving CpG or PBS alone. These results are in agreement with previous reports showing a host protective effect of i.n. immunization with *N. caninum* antigens in mice intraperitoneally challenged with this parasite.[23–25] Yet, our data extend the protective effect of i.n. immunization to *N. caninum* infection established through the gastrointestinal tract, a route more closely resembling the one naturally used for parasite penetrance into the host in horizontally transmitted neosporosis.[35] A higher number of mice without detectable parasite DNA was observed in the NcMP/CpG group than in the other studied groups. This might indicate that the immunization procedure used here could completely prevent parasite colonization in some of the infected mice. Nevertheless, further experiments will be necessary to more rigorously determine to what extent this may have happened as some of the mice that received CpG or PBS alone also presented parasite DNA below the detection limit.

CpG adjuvant typically promotes a Th1-type immune response.[37] Therefore, as could be expected, the immunization procedure assessed here induced a predominant production of antigen-specific antibodies of the IgG2a isotype, which is associated with a Th1-type immune response.[34] Nevertheless, the production of IgG1 antibodies was also raised by the i.n. immunization. As previously remarked, a balanced Th1/Th2 response might be more adequate in the course of neosporosis by conferring protection against the parasite, nonetheless avoiding fetal rejection.[25] Immunization with NcMP alone also induced the production of antigen-specific IgG. However, and in contrast with NcMP plus CpG immunization, this response was mainly characterized by the production of IgG1, indicative of a predominant Th2-type response. These results may indicate that *N. caninum* structural antigens promote a Th2-type polarization of the immune response, associated with host susceptibility to neosporosis[38] that could be overcome by the usage of CpG adjuvant. A similar effect of CpG was previously observed in mice subcutaneously immunized with *N. caninum* lysates or soluble antigen preparations.[11] Our results also reinforce the adequateness of using CpG adjuvant to achieve immunoprotection against *N. caninum* infection, as described by others using alternative immunization strategies.[11,39]

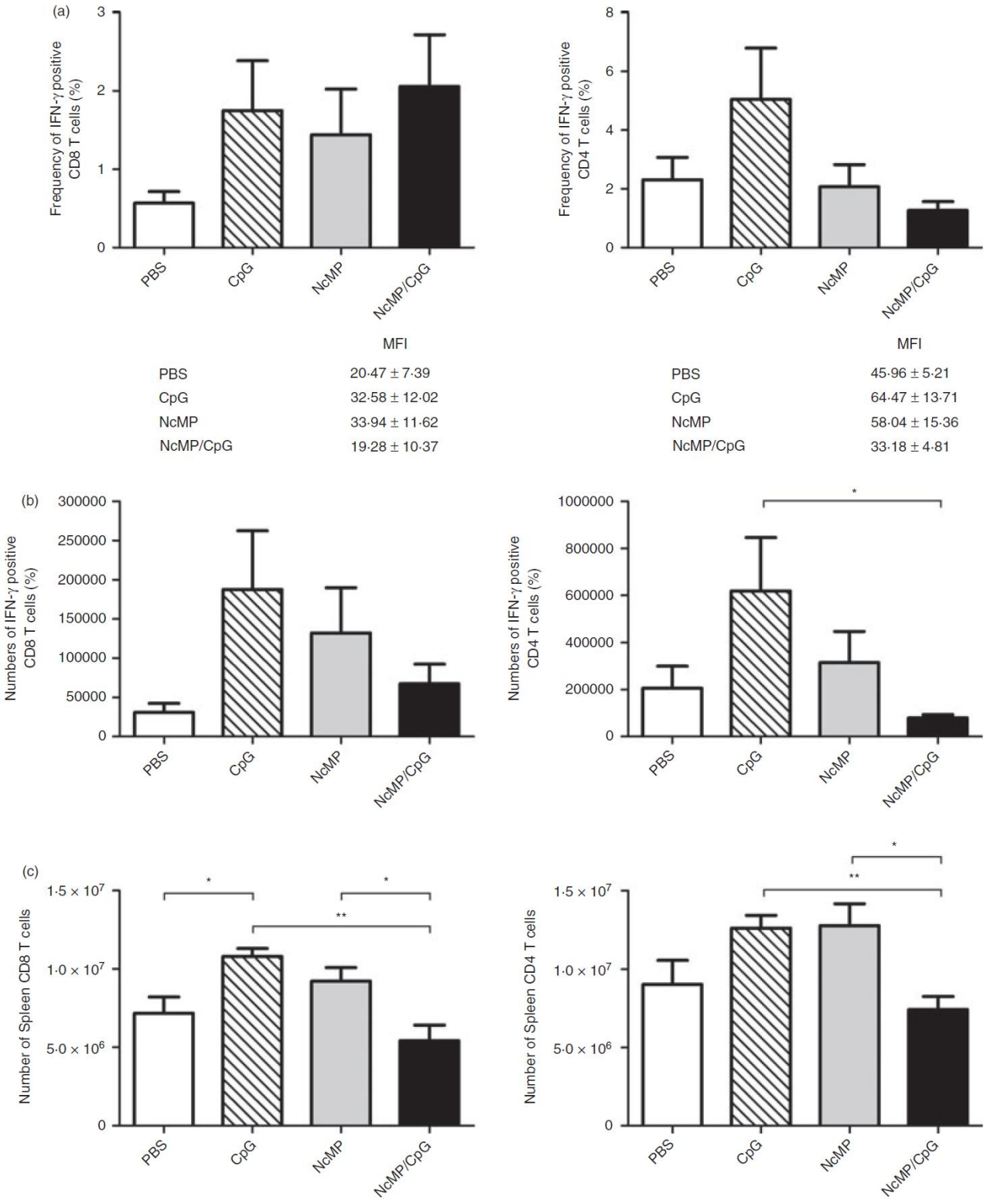

Figure 6. Frequency (a) and total numbers (b) of splenic interferon-γ-positive (IFN-γ^+) $CD8^+$ and $CD4^+$ T cells, or of (c) total splenic $CD8^+$ and $CD4^+$ T cells, detected 7 days after intragastric challenge with 5×10^7 *Neospora caninum* tachyzoites in mice previously immunized with *N. caninum* membrane proteins (NcMP) with or without CpG adjuvant (NcMP and NcMP/CpG, respectively) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG). The mean fluorescence intensity (MFI) ± one SEM due to IFN-γ staining is indicated for each group in (a). Results correspond to pooled data of two independent experiments. PBS, CpG and NcMP $n = 8$; NcMP/CpG $n = 9$. Each bar represents the mean value for each group. Error bar = SEM (*$P < 0{\cdot}05$; ** $P < 0{\cdot}01$).

Despite the fact that the serum IgG isotype profile might indicate that a Th1-type immune response was elicited in the NcMP/CpG group, no significant differences in the frequencies of splenic or MLN T cells producing IFN-γ, IL-10 and IL-4 could be detected among the studied groups. As the majority of mice from the NcMP/CpG group presented no detectable parasitic colonization in the absence of a significant increase in IFN-γ production the achieved protection could be, at least partially, independent of a strong Th1 response. Vaccine-induced protection in the absence of a Th1-type response was previously reported in mice infected by the protozoan *Leishmania amazonensis*.[40] As the parasite-specific IgA obtained from the intestinal lumen of immunized mice was shown to agglutinate *N. caninum* parasites, it might be hypothesized that the lack of a noticeable Th1-type cytokine bias in the NcMP/CpG group could result from the impairment of host invasion across the intestinal mucosa and consequent antigenic stimulation. Supporting this hypothesis, the only animal in that group that did not present detectable levels of IgA in both analysed mucosa was the one showing higher parasite DNA levels. In this scenario, although a cell mediated immune response, polarized to a Th1-type response, might be generated by the immunization, its full activation upon infection in the immunized mice could have been prevented by lack of antigenic stimulation in the NcMP/CpG group. The lack of an exacerbated cellular immune response in the spleen and MLN of the NcMP/CpG group further supports this hypothesis. As a strong production of IFN-γ in dams has previously been shown to compromise fetus viability,[41] it might be worth assessing the immunizing protocol attempted here in pregnant mice infected with *N. caninum* as we observed that protection could be attained without a marked Th1 response.

As *N. caninum* is an obligate intracellular pathogen a cell-mediated rather than a humoral immune response could be expected to be protective. Nevertheless, previous studies have reported a host protective role of intestinal IgA in cats[42] and mice[43] infected with the closely related protozoan *Toxoplasma gondii*, in agreement with the evidence presented here indicating that such a protective role may also be played by IgA in the course of *N. caninum* infection. Interestingly, non-infected mice immunized with NcMP plus CpG still presented antigen-specific intestinal IgA antibodies by 4 months after the last i.n. antigenic administration. Although preliminary, these results might indicate that this immunization procedure can induce a long-term IgA response in the gut, this would be worth assessing in more detail. Moreover, as we also show, IgG antibodies obtained from immunized mice can opsonize *N. caninum* tachyzoites and reduce parasite survival in murine macrophages challenged with this parasitic form, indicating that antibodies specific for *N. caninum* might contribute to host protection against this parasite. These results are in agreement with previous reports demonstrating that antibodies specific for different membrane proteins of *N. caninum* are capable of interfering with, and may even block, the parasite entry into host cells.[44–46] However, such a protective role needs to be confirmed in further experiments, namely as the effect of IgG opsonization on leucocyte killing of tachyzoites of the closely related protozoan *T. gondii* remains controversial with previous studies showing either enhanced killing[47] or no effect in this regard.[48]

Only a limited number of reports specifically studied the mucosal immune response to this parasite[26,27] and to the best of our knowledge no study had previously addressed the immune response in the intestinal mucosa and associated lymphoid tissue of mice immunized with *N. caninum* antigens. The widespread usage of intraperitoneal inoculation in experimental studies on neosporosis probably contributed to the overlooking of the mucosal layer of immune defence against this parasitosis. By showing that intestinal lumen antibodies induced by mucosal immunization can agglutinate *N. caninum* tachyzoites we provide the first evidence indicating that stimulating antibody production in the gut by means of mucosal immunization may be worth attempting as a protective strategy against horizontally transmitted neosporosis.

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Disclosures

The authors have no financial or any other conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Electrophoretic migration profiles of *Neospora caninum* protein extracts.

Fig. S2. Schematic representation of the time-line (in weeks) for the immunization protocol.

Fig. S3. Microscopic analysis of *Neospora caninum* tachyzoites clustering assessed by Hemacolor staining.

Fig. S4. Frequency (a) and total numbers (b) of splenic interleukin-10-positive (IL-10^+) or IL-4^+ $CD4^+$ T cells detected 7 days after intragastric (i.g.) challenge with 5×10^7 *Neospora caninum* tachyzoites in mice previously immunized with *N. caninum* membrane proteins with or without CpG adjuvant (NcMP and NcMP/CpG, respectively) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG).